Title: Development of a fast screening method for the direct
determination of chlorinated persistent organic pollutants in fish oil by
high-resolution continuum source graphite furnace molecular absorption
spectrometry

Corresponding Author: Professor Bernhard Welz, Dr.

Corresponding Author's Institution: Universidade Federal de Santa
Catarina

First Author: Ederson R Pereira, MSC

Order of Authors: Ederson R Pereira, MSC; Josias O Merib, PhD; Heloisa R
Cadorim, MSC; Mauana Schneider, MSC; Gabriel S Carvalho, MSC student;
Fabio A Duarte, PhD; Bernhard Welz, Dr.; Javier del Campo Menoyo, PhD;
Joerg Feldmann, PhD

Abstract: The occurrence of persistent organic pollutants (POPs), mainly
organochlorine pesticides and polychlorinated biphenyls, was directly
associated with several diseases and environmental endocrine disrupting.
In the aquatic environment, POPs can accumulate in fish lipoid tissues
due to their high hydrophobicity, and become this way one of the main
sources of human exposure to POPs through the consumption of fish meat
and oil as Omega-3 source. Chlorine might serve as a proxy for the
presence of POPs, and a fast screening of chlorine in a complex matrix,
such as fish oil, could provide substantial information about the
contamination with POPs. Therefore, a method has been developed in this
work for the determination of total chlorine in fish oil samples via
molecular absorption of the strontium mono-chloride molecule in the gas
phase using high-resolution continuum source graphite furnace molecular
absorption spectrometry. The effect of zirconium as permanent chemical
modifier in the pyrolysis and vaporization stages was optimized in order
to avoid the need for any kind of sample preparation prior to the
determination of total chlorine, using just a dilution with 1-propanol.
The accuracy has been evaluated using micro-coulometric titration after
sample combustion, and the values were statistically in agreement (95%
confidence level) between both techniques. The method has been applied
for the determination of total chlorine in five different fractions of a
commercial pooled marine fish oil sample collected from the Pacific
Ocean, where the majority of the fish is Peruvian anchovy (Engraulis
ringens), two commercial oils from Brazil and three Omega-3 supplements
acquired in Germany. The limit of detection of the procedure is 1.8 ng Cl
absolute or 0.9 µg g-1 Cl in the fish oil. The time required for a single
determination is less than 5 min, and less than 15 min for a triplicate
determination.

UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS FÍSICAS E MATEMÁTICAS
DEPARTAMENTO DE QUÍMICA
Prof. Dr. Bernhard Welz
88040-900 - Florianópolis - SC - Brasil
Phone: +55 48 3733 8876 - Fax: +55 48 3721-6850 - E-mail: w.bernardo@terra.com.br
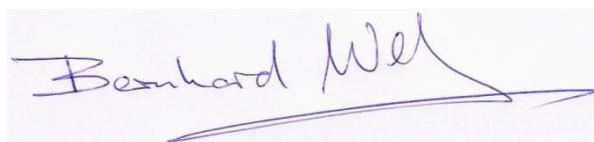
Florianópolis, 11 March, 2017

Dear Editor,

Attached please find our revised manuscript entitled: "Development of a fast screening method for the direct determination of chlorinated persistent organic pollutants in fish oil by high-resolution continuum source graphite furnace molecular absorption spectrometry". All the comments of the Reviewers have been considered in the revised manuscript, and the English has been carefully revised as well. We hope that the manuscript can now be accepted for publication in FOOD CONTROL.

I would like to declare in the name of all co-authors that the work described is original and has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the consent of the copyright holder. I also declare that there is no potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could influence of being perceived to influence our work.

Kind regards,

Prof. Dr. Bernhard Welz

Éderson R. Pereira et al.: **Development of a fast screening method for the direct determination of chlorinated persistent organic pollutants in fish oil by high-resolution continuum source graphite furnace molecular absorption spectrometry**

Response to Reviewers' comments.

Reviewer #1: The comments for the manuscript FOODCONT-D-17-00241 by Pereira et al The manuscript describes the application of HR-CS MAS for the determination of total chlorine via SrCl in fish oil which might serve as a proxy for the presence of persistent organic pollutants (POPs).
The use of SrCl fort he determination of Cl via HR-CS MAS was first proposed in a previous paper by Pereira et al. It is a useful and novel technique for total Cl determiantionwtth many advantages stated in the manuscript and this manuscript is an interesting application. I have no basic comment but I raised some specific comments hoping that they would be helpful to improve the paper. I proposed that it should be published after revisions which are itemized in the following as well as indicated on the orginal manuscript as annotations (sticky notes, deletions, corrections, questions, suspections, additions etc) .

1. English of the manuscript should be re-considered. There are some grammatical errors. I believe the authors (corresponding and the others) would do it successfully in the last version. I proposed some corrections on the manuscript attached but there are more than I did in the manuscript.

Response: The English has been carefully revised, and we hope that all errors and mistakes have now been eliminated.

2. p.3 lines 26-28: I suspect that the chlorine concentrations in fish oils may an exact indication of POPs. Chlorine may be orginated from other sources and nature of the fish used for fish-oil production. If you have a reliable literature, please give it in the introduction section.

Response: The potential falsification of the results due to chloride and polar organic chlorine compounds has been excluded using an extraction of several fish oil samples with water, which showed that there was no extractable chlorine in the samples.

3. P.9 -10 subsection "3.4. Application and evaluation of possible chlorine sources". I am convinced that the accuracy of the method is satisfactory. However, the explanation for the evaluation of possible chlorine sources in Table 3 is rather weak for me. This part should be stressed and more clarified.

Response: The accuracy of the developed method has been tested using an independent analytical technique and is quite satisfactory. The explanation of the reasons for the different chlorine concentrations found in the different samples has been given by one of the co-authors, who has a profound knowledge of the procedures used in the production, cleaning and concentration of fish oil, and their effect on the chlorine content in the resulting product.

4. p.14 line 408: I think the reference is not correct. It should be "Ozbek,N & Akman,S. (2016) Determination of Chlorine in Milk via Molecular Absorption of SrCl Using High-Resolution Continuum Source Graphite Furnace Atomic Absorption Spectrometry, J. Agric. Food Chem. 64, 5767–5772 "

Response: Thank you for pointing on this mistake; the reference has been corrected accordingly.

5. All comments were indicated on the text attached.
As a result, I propose that the manuscript should be published in Food Control after some minor revisions.

Response: Thank you for your useful comments.

Reviewer #2: The paper by Pereira et al. demonstrates an interesting application of high-resolution continuum source graphite furnace molecular absorption spectrometry, which is a powerful technique that enables the determination of traces of non-metals, such as Cl, in a large variety of samples. The MS is clear and concise, the application is well chosen, and the results reported are relevant for the community, so I can recommend its publication in Food Control.

I have only a few minor suggestions to make:

There a couple of mistakes with the references. In particular, in page 4, when referring to determination of chlorine, the reference to Ozbek & Akman, 2016 does not seem to be correct, since such paper is devoted to fluorine determination. Instead, as the authors as referring to recent articles, there a few ones focused on Cl determination that could be cited: Talanta Volume 162, 1 January 2017, Pages 354-361; Microchemical Journal, Volume 132, May 2017, Pages 130-135; J. Anal. At. Spectrom., 2015, 30, 1531-1540.

Response: The reference has been corrected, and the new references included, as proposed.

In page 8, the same occurs. The authors mention determination of Cl in milk by Ozbek & Akman in 2015, but the reference corresponds to a determination of fluorine (and in wine, not in milk). I think the authors are probably willing to cite Journal of Agricultural and Food Chemistry 64 (28), 5767-5772 instead.

Response: The reference has been corrected accordingly.

I would suggest merging Figures 1 and 2 into a single figure, and adding an additional figure showing the 3D SrCl spectrum obtained for a sample.

Response: The two Figures 1 and 2 have been merged and are now Fig. 2a and 2b; a new Fig. 1 with a 3D spectrum of a fish oil has been added.

1 **Development of a fast screening method for the direct determination of**

2 **chlorinated persistent organic pollutants in fish oil by high-resolution**

3 **continuum source graphite furnace molecular absorption spectrometry**

4 *Éderson R. Pereira [a], Josias Merib [a], Heloisa R. Cadorim [a], Mauana Schneider [a],*

5 *Gabriel S. Carvalho [b], Fabio A. Duarte [b], Bernhard Welz [a,c]*, Javier del Campo*

6 *Menoyo [d], Joerg Feldmann [e]*

7 [a] Departamento de Química, Universidade Federal de Santa Catarina, Campus Trindade,

8 88040-900 Florianópolis - SC, Brazil

9 [b] Departamento de Química, Universidade Federal de Santa Maria, 97105-900 Santa

10 Maria - RS, Brazil

11 [c] Instituto Nacional de Ciência e Tecnologia do CNPq – INCT de Energia e Ambiente,

12 Universidade Federal da Bahia, Campus Ondina, Salvador – BA, Brazil

13 [d] Golden Omega S.A., Av. Comandante San Martin 3460, Lote B-2, Arica, Chile

14 [e] Trace Element Speciation Laboratory (TESLA), Department of Chemistry, University

15 of Aberdeen, Scotland, UK

16 *Corresponding Author, e-mail address: w.bernardo@terra.com.br (B.Welz).

17 Fax: +55 48 3721 6850, phone: +55 48 3733 8876

18

Abstract

The occurrence of persistent organic pollutants (POPs), mainly organochlorine pesticides and polychlorinated biphenyls, was directly associated with several diseases and environmental endocrine disrupting. In the aquatic environment, POPs can accumulate in fish lipoid tissues due to their high hydrophobicity, and become this way one of the main sources of human exposure to POPs through the consumption of fish meat and oil as Omega-3 source. Chlorine might serve as a proxy for the presence of POPs, and a fast screening of chlorine in a complex matrix, such as fish oil, could provide substantial information about the contamination with POPs. Therefore, a method has been developed in this work for the determination of total chlorine in fish oil samples via molecular absorption of the strontium mono-chloride molecule in the gas phase using high-resolution continuum source graphite furnace molecular absorption spectrometry. The effect of zirconium as permanent chemical modifier in the pyrolysis and vaporization stages was optimized in order to avoid the need for any kind of sample preparation prior to the determination of total chlorine, using just a dilution with 1-propanol. The accuracy has been evaluated using micro-coulometric titration after sample combustion, and the values were statistically in agreement (95% confidence level) between both techniques. The method has been applied for the determination of total chlorine in five different fractions of a commercial pooled marine fish oil sample collected from the Pacific Ocean, where the majority of the fish is Peruvian anchovy (*Engraulis ringens*), two commercial oils from Brazil and three Omega-3 supplements acquired in Germany. The limit of detection of the procedure is 1.8 ng Cl absolute or 0.9 µg g^{-1} Cl in the fish oil. The time required for a single determination is less than 5 min, and less than 15 min for a triplicate determination.

Keywords: Persistent organic pollutants; Organochlorines; Total chlorine determination; Fish oil; Omega-3 supplements; High-resolution continuum source molecular absorption spectrometry.

1. Introduction

Chlorine has a huge importance in human health and may be present in several matrices with very different degrees of complexity. Some of the chlorine compounds, known as "organochlorines", are a class of the "Persistent Organic Pollutants" (POPs) and have properties of accumulation in lipid-rich tissues and sediments (Merib, Nardini, & Carasek, 2014). Therefore, the bioaccumulation of chlorine in fish tissues in aquatic systems emerges as a possible contamination, once fish is an important component of the diet of many people around the world. In addition, the oil obtained from fish is a source of energy and calories, a mixture of colorants, steroids, glycids, phospholipids and fatty acids, where polyunsaturated Omega-3 fatty acids can be found, predominantly with four to six double bonds (Pereira et al., 2016).

During the industrial processes leading to the purification of the raw fish oil and concentration of the Omega-3 fatty acids, the concentration of the organochlorine compounds is lowered to meet regulatory specifications, relying on chromatographic methods for the individual quantification of compounds or classes of compounds in the final product. However, the level of complexity and cost required for such determinations, including sample preparation steps, precludes their application in the quality control of an industrial laboratory. Considering that most of the regulated POPs contain chlorine in their molecules, the determination of total chlorine in the oil with little or no sample preparation can be envisaged as an excellent tool in a manufacturing environment.

Usual monitoring methods are gas chromatography with mass spectrometric detection (GC-MS) and to a lesser extent high performance liquid chromatography electron spray ionization mass spectrometry (HPLC-ESI-MS). Both techniques are molecular-specific and are useful if detailed information about the nature of the chlorinated compounds is needed. However, they are often used for target analysis and not giving the total sum of all chlorinated compounds since they are hidden amongst hundreds of other organic compounds, which are in much higher concentrations. Additionally, due to the use of chromatographic separation the sample throughput is not high, leaving alone the need for sample preparation steps.

A variety of methods was proposed to determine chlorine in different matrices, including classical procedures, such as gravimetric or volumetric analysis, ion-chromatography and ion-selective electrode potentiometry (Flores et al., 2008; Mello et al., 2013; Peng, Wu, Lai, Xiao, & Li, 2012; Smith, McMurtrie, & Galbraith, 1977).

However, these techniques often require a sample digestion prior to analyte determination, including Schöniger oxidation (Flores, Barin, Mesko, & Knapp, 2007), alkaline fusion (Blackwell, Cave, Davis, & Malik, 1997), pyrohydrolysis (Duarte et al., 2013) or microwave-induced combustion (Flores et al., 2007; Flores et al., 2008). Plasma-based techniques, such as inductively coupled plasma optical emission spectrometry (ICP OES) and mass spectrometry (ICP-MS) are not usually employed for the determination of chlorine. In the case of ICP OES, the wavelengths of this element are situated in the vacuum-UV (< 200 nm), making it difficult to separate the analytical signal from the noise, unless a purged monochromator is used (Welz et al., 2009). On the other hand, the ionization is also very low due to the high ionization potential of Cl (12 eV) in an argon-based plasma.

Eliminating sample preparation as much as possible from the analytical protocol avoids or at least reduces the risk of contamination and analyte losses, is less time consuming, and often improves the limit of detection. However, only a few analytical techniques have shown some capacity for the determination of chlorine with relative sensitivity and accuracy, mainly for complex matrices, using direct determination. Among these techniques are electrothermal vaporization inductively coupled plasma mass spectrometry (ETV-ICP-MS) (Antes et al., 2013; Gois, Pereira, Welz, & Borges, 2014; Gois, Pereira, Welz, & Borges, 2015), laser induced plasma spectrometry (LIPS) (Kaski, Häkkänen, & Korppi-Tommola, 2004), X-ray fluorescence spectrometry (XRF) (Doyle, Saavedra, Tristão, Nele, & Aucélio, 2011), and more recently also high-resolution continuum source graphite furnace molecular absorption spectrometry (HR-CS GF MAS) (Bechlin, Ferreira, & Gomes Neto, 2017; Enders et al., 2016; Guarda et al., 2017; Heitmann, Becker-Ross, Florek, Huang, & Okruss, 2006; Nakadi, da Veiga, Aramendia, Garcia-Ruiz, & Resano, 2015; Ozbek & Akman, 2016; Pereira et al., 2015; Pereira et al., 2014; Welz, Vale, Pereira, Castilho, & Dessuy, 2014). The last technique is a very robust tool with a high tolerance for complex matrices due to the use of a graphite tube furnace, which makes possible the analysis of liquids, slurries and solid samples. It often permits to skip sample preparation, making this technique an interesting alternative for the determination of chlorine.

For MAS, bands of diatomic molecules that exhibit a pronounced rotational fine structure can be formed in the graphite tube vaporizer employing a molecule-forming reagent and can be monitored for quantitative determination (Welz et al., 2009). The successful application of HR-CS GF MAS is correlated with the continuum radiation

115 source coupled to a high-resolution double monochromator and a linear charge-coupled
116 device (CCD) array detector providing a resolution of $\lambda/\Delta\lambda \approx 175,000$, which makes
117 possible the use of the entire spectral region (190 - 900 nm) for analytical measurement
118 at high resolution (Welz, 2004; Welz et al., 2014). The diatomic molecules formed in
119 the gas phase should have dissociation energies higher than 400 kJ mol^{-1} to ensure their
120 stability at the temperatures of the pyrolysis and vaporization stages, and avoid
121 formation of competitive molecules (Butcher, 2013).

122 The goal of this work was to develop a fast and simple procedure for the direct
123 determination of chlorine in fish oil samples via the SrCl molecule using HR-CS GF
124 MAS, so that a screening for all organochlorine compounds in a fish oil sample
125 becomes feasible. The method describes the use of strontium carbonate solution as the
126 molecule-forming reagent and Zr as permanent chemical modifier, investigating
127 different parameters, such as pyrolysis and vaporization temperatures in relation to
128 matrix components that could interfere directly with the stability of the molecule.

129 **2. Experimental**

130 *2.1. Instrumentation*

131 All measurements have been made using a high-resolution continuum source
132 atomic absorption spectrometer Model contrAA 600 (Analytik Jena AG, Jena,
133 Germany). It is equipped with a transversely heated graphite tube atomizer and a xenon
134 short-arc lamp as the radiation source, with emits a spectral continuum between 190 and
135 900 nm. The spectrometer consists of a high-resolution double monochromator,
136 equipped with a prism pre-monochromator for pre-dispersion of the radiation and an
137 echelle grating monochromator for the high resolution. The analytical signal is detected
138 using a CCD array detector with 588 pixels, 200 of which are used for analytical
139 purposes, displaying the vicinity of the analytical line at high resolution (ca. 1.5 pm /
140 pixel at 200 nm).

141 Chlorine has been determined via the molecular absorption of SrCl (Pereira et al.,
142 2014), which has been measured at 635.862 nm (Figure 1) using the integrated
143 absorbance of three pixels (peak volume selected absorbance, PVSA, $A_{\Sigma3,int}$) (Heitmann,
144 Welz, Borges, & Lepri, 2007). All measurements were carried out using pyrolytically
145 coated graphite tubes with PIN platform (Analytik Jena Part No. 407-A81.025) and a
146 sample volume of 20 µL injected with a micro-pipette. Argon 99.996% (Air Liquid,
147 Florianópolis, Brazil) was used as a purge and protective gas. The optimized

5

148 temperature program used for all determinations with HR-CS GF MAS, is shown in
149 Table 1.

150   Chlorine was also determined by micro-coulometric titration after sample
151 combustion in a Cl analyser (Model Multi EA® 5000 elemental analyzer, Analytik
152 Jena), using Ag/AgCl and Pt electrodes. Fish oil samples were weighed and introduced
153 directly into the combustion tube by a solid sample introduction system (Model Multi-
154 matrix sampler MMS 5000, Analytik Jena), using a quartz boat (40 x 9 mm, Part No.
155 402-889.674, Analytk Jena). The operational conditions of the Cl analyser were used as
156 recommended by the manufacturer: furnace temperature: 1050 °C, time for second
157 combustion: 90 s, oxygen flow rate: 100 mL min^{-1} and argon flow rate: 100 mL min^{-1}.
158 Results were measured in peak area with 20 min integration time.

159 *2.2. Reagents and materials*

160   All reagents used for this purpose presented at least analytical grade of purity.
161 Ultrapure water (resistivity 18 MΩ cm) was obtained from a model Mega ROUP
162 purification system (Equisul, Pelotas, Brazil) and was used for preparation of the
163 standard solutions of 1000 mg L^{-1} Cl prepared by dissolving appropriate amounts of
164 NaCl (Fluka, Buchs, Switzerland). A stock solution of 10 g L^{-1} Sr^{2+} was prepared by
165 dissolving appropriate amounts of SrCO$_3$ (Vetec, Duque de Caxias, Brazil) and was
166 used as the molecule-forming reagent. The solution of 1 g L^{-1} Zr (Fluka) was used as
167 permanent chemical modifier. All bottles were decontaminated with 10% v/v nitric acid
168 (Merck, Darmstadt, Germany) for 24 hours and then rinsed with ultrapure water three
169 times before use.

170 *2.3. Procedure*

171 *2.3.1. Direct HR-CS GF MAS determination*

172   When HR-CS GF MAS was used, zirconium was initially deposited on the PIN-
173 platform in the graphite tube using ten aliquots of 40 μL of the 1 g L^{-1} Zr solution, each
174 injection followed by a five-step temperature program with previously optimized ramp
175 and hold times after each injection, resulting in a platform coated with 400 μg Zr
176 (Pereira et al., 2014). Subsequently, about 0.4 g of fish oil were diluted to 2 mL with 1-
177 propanol and 20 μL of this diluted sample were pipetted onto the Zr-treated PIN-
178 platform in the graphite tube and the formation of the SrCl molecule in the vaporization

179 stage was stimulated by adding 10 µL of a 1% (m/v) Sr^{2+} solution and the absorbance

180 measured as described in Section 2.1.

2.3.2. Micro-coulometric procedure

182 For micro-coulometric titration after sample combustion, a sample mass between

183 15 and 111 mg was directly weighed on a quartz boat and introduced into the

184 combustion tube using the solid sampling device of the equipment. After sample

185 combustion, the reaction products were transferred through the gas transfer line to the

186 micro-coulometric titration system for Cl determination. The instrument was calibrated

187 by injection of Cl standards using an automatic syringe, an accessory available for the

188 instrument. The calibration range was from 0.8 to 4.0 µg Cl (chlorobenzene diluted in

189 toluene), with R^2 better than 0.999. The accuracy of the method was evaluated using the

190 certified reference material BCR 181 (coking coal), with a certified concentration of

191 1.38 ± 0.05 mg g^{-1}, and a concordance better than 95% (Student's t-test) has been

192 obtained.

2.3.3. Liquid-liquid extraction

194 In order to eliminate the possibility that chloride or other polar chlorinated

195 compounds are in the lipophilic samples as micelles and increase the measured value for

196 POPs erroneously, a liquid/liquid extraction was performed using ultrapure water and

197 oil sample. Approximately 0.5 g of fish oil was extracted using 0.5 mL ultrapure water,

198 shaken for 1 min and centrifuged at 3000 r.p.m for 10 min. The aqueous extract was

199 separated and 20 µL of this extract was injected onto the graphite platform for the

200 determination of chorine using HR-CS GF MAS. The analytical signal obtained for the

201 aqueous extract was compared with the blank solution, using just the tube with the Zr

202 coated platform and 10 µL of the 1% (m/v) Sr^{2+} solution for molecule formation.

2.4. Fish oil samples

204 The crude oil was extracted from fish from the Pacific Ocean, the majority of which

205 being Peruvian anchovy (*Engraulis ringens*) and Chilean mackerel (*Trachurus*

206 *murphyi*). The samples were kept in polyethylene flasks at room temperature until they

207 were analyzed. The samples were named as follows: Raw fish oil; Bandolado fish oil;

208 Crude fish oil; Waste fraction (dilute omega-3); Intermediate product. These samples

209 correspond to different stages of the purification process, according to the extraction

210 and purification of marine fish oil by Golden Omega S.A. (Arica, Chile). Other samples

named here as sample A and sample B were acquired at a local supermarket in Florianópolis, SC, Brazil and are classified as brute oil (no clean-up stages applied). Additionally three commercial oils 1-3 were acquired at a drugstore in Jena, Germany, and have been classified as pure oil containing polyunsaturated Omega-3 fatty acids and were used as samples.

3. Results and discussion

3.1. Optimization of pyrolysis and vaporization temperatures

Chlorine was determined successfully in several different samples using direct analysis via the absorption of the SrCl molecule at 635.862 nm in the gas phase of a graphite tube furnace when vaporization temperatures between 1800 ℃ and 2400 ℃ were used. The first method employing aqueous strontium as molecule-forming reagent (10 µL of 1% (m/v) Sr^{2+} solution; 0.10 mg Sr^{2+}), for the determination of chlorine was described by Pereira et al. (Pereira et al., 2014). It was applied for the direct determination of total chlorine in biological samples using Zr as permanent chemical modifier and a PIN-platform tube. Later studies have adapted successfully this methodology for the direct determination of chlorine in coal (Pereira et al., 2015), crude oil (Enders et al., 2016) and milk (Ozbek & Akman, 2016), also using direct analysis. This demonstrates the wide application field for this methodology, highlighting also the robustness of the direct analysis approach compared to other methods that use more elaborate sample preparation. The crucial parameters for this present work, such as permanent modifier (zirconium), graphite tube with PIN-platform, and a strontium mass of 0.10 mg were therefore fixed according to previous optimization (Pereira et al., 2015; Pereira et al., 2014), and the pyrolysis and vaporization temperatures were established comparing the integrated absorbance and the analytical signal of chlorine in fish oil and in an aqueous standard solution.

The pyrolysis curves for chlorine in an aqueous solution containing 15 ng Cl and in 10 µL of raw fish oil are shown in Figure 2(a). The analytical signal remains constant for temperatures between 900 ℃ and about 1300 °C for chlorine in fish oil and in aqueous solution. Temperatures lower than 900 °C were not considered due to the very noisy analytical signal in the case of the fish oil sample, due to the dense smoke caused by the residual lipoid matrix in the tube at these temperatures. The pyrolysis curve found in this work for an aqueous chlorine standard solution was consistent with that found in earlier work (Pereira et al., 2015; Pereira et al., 2014), with pyrolysis

244   temperatures of up to 1300 °C without loss of sensitivity. As the same high temperature

245   can be used for the fish oil samples, it can be made sure that essentially the entire matrix

246   can be eliminated. The similarity between the behavior of Cl from fish oil and an

247   aqueous standard solution makes possible using 1300 °C as optimal pyrolysis

248   temperature.

249   The vaporization curves for Cl (as SrCl) in the aqueous standard solution and in the

250   fish oil sample are shown in Figure 2(b) for the temperature range 1700 °C - 2400 °C.

251   The maximum molecular absorbance signal was obtained for the aqueous standard

252   solution and for the fish oil when temperatures of 2000 °C and 2100 °C were used. The

253   results for the aqueous standard agreed with those found in earlier work (Pereira et al.,

254   2014), where a similar profile was obtained using Zr as a permanent chemical modifier

255   and a graphite tube with integrated PIN-platform. A vaporization temperature of 2100

256   °C was chosen for all future work.

3.3. Calibration and figures of merit

258   The figures of merit, such as calibration range, limit of detection (LOD) and

259   quantification (LOQ) using aqueous standard solutions for calibration were evaluated

260   and are shown in Table 2. The LOD and LOQ were calculated as three and ten times the

261   standard deviation of 10 blank measurements (10 µL of 1% m/v Sr^{2+} solution; 0.10 mg

262   Sr^{2+}) divided by the slope of the calibration curve, 3 and $10\sigma/S$, considering 0.4 g of

263   sample diluted to 2 mL using 1-propanol. The characteristic mass (m_o) was calculated

264   as $A_{int}= 0.0044$ s/S, S as 0.0136 s ng^{-1}. These two parameters, the LOD of 1.8 ng and m_0

265   of 0.32 ng found in this work were largely consistent with the values of 0.85 and 0.24

266   ng, found in earlier work (Pereira et al., 2015); however, they were lower than those of

267   1.0 ng and 2.2 ng, respectively, found by Pereira et al. (Pereira et al., 2014) for Cl in

268   biological samples. This difference is according to expectation, as in the latter work a

269   compromise temperature was used, which changed the working range and therefore also

270   the figures of merit.

3.4. Application and evaluation of possible chlorine sources

272   The developed method was applied for the determination of total chlorine in ten

273   fish oil samples, using the absorption of the SrCl molecule formed in the gas phase of a

274   graphite tube furnace, and the results are shown in Table 3. In order to compare the

275   results found by HR-CS GF MAS, total chlorine was also determined in five samples

276　(raw, bandolado, crude, sample A, and sample B) using micro-coulometric titration, and

277　the results were not significantly different between the two methods (Student's t-test).

278　Among the fish oil samples analyzed with the method, the first one (raw oil) showed the

279　30highest concentration of chlorine (56.9 ± 2.8 µg g^{-1}) compared to the samples that

280　underwent the cleaning process (intermediate product), the chlorine concentration of

281　which was 3.5 ± 0.2 µg g^{-1}. Comparing the results found for raw, bandolado ($.3 \pm 0.5$ µg

282　g^{-1}) and crude oil (26.4 ± 0.5 µg g^{-1}), the efficiency of already the first cleaning process

283　as decontaminant becomes obvious, once about 50% of the chlorine was eliminated.

284　　　The low concentration of chlorine in the intermediate product reflects the high

285　efficiency of the subsequent process steps employing controlled heating after

286　purification using the inorganic adsorbent. Obviously, a mass balance is not benefited

287　here due to the losses of chlorine caused by the volatilization and/or diffusion of them

288　and a different concentration might be found in the waste fraction (31.1 ± 2 µg g^{-1}). The

289　samples A and B, obtained from a local supermarket, showed a similar concentration of

290　chlorine (about 11.5 ± 0.9 µg g^{-1} Cl), and these results were consistent with those found

291　when using micro-coulometric titration.

292

293　*3.4.1. Liquid-liquid extraction*

294　　　In order to make sure that the chlorine measured in this direct determination is not

295　coming from ionic forms distributed throughout the fish oil sample, a liquid-liquid

296　extraction using the raw fish oil was performed using water as extractor. The aqueous

297　phase was directly injected onto the platform of the graphite furnace for chlorine

298　determination as SrCl. In this way, if the fish oil would contain such ionic inclusions,

299　chlorine would migrate to the water phase and be detected. So, it would not be possible

300　using the proposed method for a fast screening for chlorinated POPs. However, no

301　difference in the analytical signal was found comparing the water phase from liquid-

302　liquid extraction and the blank solution submitted to the same method of determination.

303　Hence, this is a substantial information that chlorine composes organic molecules or

304　lipoid structures, which are in high concentration in oil samples (Pereira et al., 2016),

305　and that the proposed direct method can be used for a fast screening for chlorinated

306　POPs.

307　*3.4.2. Further evaluation of the commercial fish oil capsules*

10

308   In order to understand better the three commercial fish oil samples and the

309   difference in their chlorine content, we first of all consulted the content of Omega-3

310   fatty acids, principally docosahexaenic acid (DHA) and eicosapentaenic acid (EPA),

311   claimed on the label of the capsules, which is shown in Table 4. We also compared the

312   color of the three samples, which is shown in Figure 4, and which might give some

313   insight into the cleaning process that was used for the fish oil. Raw fish oil contains

314   about 300 mg g^{-1} Omega-3 fatty acids with a typical ratio of some 180 mg g^{-1} EPA and

315   120 mg g^{-1} DHA.

316   It is obvious from the concentration of DHA + EPA shown in Table 4 that all three

317   products were fish oil, not concentrated fish oil. As sample No. 1 has a darker color

318   compared to the other samples, it has probably not gone through a distillation process,

319   but only through a simple treatment with bleaching clays to remove mucilages, colloids

320   and also arsenic and heavy metals etc. The high chlorine content of 17 µg g^{-1} in this

321   sample is consistent with the manufacturing process, which obviously did not include

322   any distillation. As POPs have a higher boiling point than Omega-3 fatty acids, they

323   remain in the residue of the distillation process and are removed from the fish oil

324   fraction. Samples No. 2 and 3, in contrast, have gone through a distillation, which is

325   clearly visible from their lighter color, but only to remove POPs, not to increase the

326   Omega-3 concentration. Sample No. 2 actually has a lower content of omega-3 fatty

327   acids than normal, and it might be suspected that it is a head fraction of the distillation

328   process. In summary, the total chlorine determination appears to be an excellent marker

329   of processing steps of the fish oil and might replace complicated and time-consuming

330   chromatographic measurements in a routine lab.

331   **4. Conclusion**

332   The present work demonstrates the use of high-resolution continuum source

333   graphite furnace molecular absorption spectrometry (HR-CS GF MAS) for the

334   determination of chlorine in fish oil. The absorbance of the SrCl molecule formed in the

335   gas phase of the graphite tube furnace upon the addition of SrCO$_3$ and heating is an

336   excellent tool for the sensitive determination of chlorine in complex matrices.

337   Zirconium was used as a permanent chemical modifier on the PIN platform in the

338   graphite tube and just a moderate dilution with 1-propanol was employed in order to

339   decrease the high viscosity of fish oil. The developed method can be classified as fast

340   and simple, once no other reagents are added and no sample pre-treatment whatever is

necessary. Therefore it is ideally suited for the control of the POPs removal process during the manufacturing of Omega-3 concentrates. This direct analysis approach is also a significant contribution to the accuracy of the procedure, as chlorine can be considered an omnipresent element, and any kind of sample pre-treatment, particularly digestion procedures, might become a source of contamination and error. The good accuracy and precision obtained in this work proves the absence of any significant contamination. The proposed method showed good agreement with the results of an alternative micro-coulometric titration technique (higher than 90%). This and the fact that aqueous standard solutions could be used for calibration further demonstrate the absence of any kind of interference. The determination of total chlorine has been shown to be an excellent marker of the proper removal of POPs during the purification/concentration steps by state-of-the-art molecular distillation, and of the processing steps in general. It might be speculated that this direct analysis approach might be applicable, maybe with some modification, for the determination of other analytes in fish oil and similar lipid matrices.

Acknowledgement

References

Antes, F. G., Dullius, E., da Costa, A. B., Molz, R. F., Paniz, J. N. G., Flores, E. M. M., & Dressler, V. L. (2013). Development of a vaporization system for direct determination of chlorine in petroleum coke by ICP-MS. *Microchemical Journal, 109*, 117-121.

Bechlin, M. A., Ferreira, E. C., & Gomes Neto, J. A. (2017). Determination of chlorine in cement via CaCl molecule by high-resolution continuum source graphite furnace molecular absorption spectrometry with direct solid sample analysis. *Microchemical Journal, 132*, 130-135.

Blackwell, P. A., Cave, M. R., Davis, A. E., & Malik, S. A. (1997). Determination of chlorine and bromine in rocks by alkaline fusion with ion chromatography detection. *Journal of Chromatography A, 770*, 93-98.

Butcher, D. J. (2013). Molecular absorption spectrometry in flames and furnaces: A review. *Analytica Chimica Acta, 804*, 1-15.

Doyle, A., Saavedra, A., Tristão, M. L. B., Nele, M., & Aucélio, R. Q. (2011). Direct chlorine determination in crude oils by energy dispersive X-ray fluorescence spectrometry: An improved method based on a proper strategy for sample homogenization and calibration with inorganic standards. *Spectrochimica Acta - Part B Atomic Spectroscopy, 66*, 368-372.

Duarte, F. A., Pereira, E. R., Flores, E. L. M., Muller, E. I., Flores, E. M. M., & Dressler, V. L. (2013). Spectrophotometric determination of chloride in cement after sample preparation by pyrohydrolysis. *Quimica Nova, 36*, 716-719.

Enders, M. S. P., Gomes, A. O., Oliveira, R. F., Guimaraes, R. C. L., Mesko, M. F., Flores, E. M. M., & Müller, E. I. (2016). Determination of Chlorine in Crude Oil by High-Resolution Continuum Source Graphite Furnace Molecular Absorption Spectrometry Using AlCl, InCl, and SrCl Molecules. *Energy and Fuels, 30*, 3637-3643.

Flores, É. M. M., Barin, J. S., Mesko, M. F., & Knapp, G. (2007). Sample preparation techniques based on combustion reactions in closed vessels — A brief overview and recent applications. *Spectrochimica Acta Part B: Atomic Spectroscopy, 62*, 1051-1064.

Flores, É. M. M., Mesko, M. F., Moraes, D. P., Pereira, J. S. F., Mello, P. A., Barin, J. S., & Knapp, G. (2008). Determination of Halogens in Coal after Digestion

Using the Microwave-Induced Combustion Technique. *Analytical Chemistry, 80*, 1865-1870.

Gois, J. S., Pereira, É. R., Welz, B., & Borges, D. L. G. (2014). Simultaneous determination of bromine and chlorine in coal using electrothermal vaporization inductively coupled plasma mass spectrometry and direct solid sample analysis. *Analytica Chimica Acta, 852*, 82-87.

Gois, J. S., Pereira, É. R., Welz, B., & Borges, D. L. G. (2015). Application of direct solid sample analysis for the determination of chlorine in biological materials using electrothermal vaporization inductively coupled plasma mass spectrometry. *Spectrochimica Acta - Part B Atomic Spectroscopy, 105*, 12-17.

Guarda, A., Aramendía, M., Andrés, I., García-Ruiz, E., do Nascimento, P. C., & Resano, M. (2017). Determination of chlorine via the CaCl molecule by high-resolution continuum source graphite furnace molecular absorption spectrometry and direct solid sample analysis. *Talanta, 162*, 354-361.

Heitmann, U., Becker-Ross, H., Florek, S., Huang, M. D., & Okruss, M. (2006). Determination of non-metals via molecular absorption using high-resolution continuum source absorption spectrometry and graphite furnace atomization. *Journal of Analytical Atomic Spectrometry, 21*, 1314.

Heitmann, U., Welz, B., Borges, D. L. G., & Lepri, F. G. (2007). Feasibility of peak volume, side pixel and multiple peak registration in high-resolution continuum source atomic absorption spectrometry. *Spectrochimica Acta Part B: Atomic Spectroscopy, 62*, 1222-1230.

Kaski, S., Häkkänen, H., & Korppi-Tommola, J. (2004). Determination of Cl/C and Br/C ratios in pure organic solids using laser-induced plasma spectroscopy in near vacuum ultraviolett. *Journal of Analytical Atomic Spectrometry, 19*, 474-478.

Mello, P. A., Barin, J. S., Duarte, F. A., Bizzi, C. A., Diehl, L. O., Muller, E. I., & Flores, E. M. M. (2013). Analytical methods for the determination of halogens in bioanalytical sciences: a review. *Analytical and Bioanalytical Chemistry, 405*, 7615-7642.

Merib, J., Nardini, G., & Carasek, E. (2014). Use of Doehlert design in the optimization of extraction conditions in the determination of organochlorine pesticides in bovine milk samples by HS-SPME. *Analytical Methods, 6*, 3254-3260.

431 Nakadi, F. V., da Veiga, M. A. M. S., Aramendia, M., Garcia-Ruiz, E., & Resano, M.
432   (2015). Chlorine isotope determination via the monitoring of the AlCl molecule
433   by high-resolution continuum source graphite furnace molecular absorption
434   spectrometry - a case study. *Journal of Analytical Atomic Spectrometry, 30*,
435   1531-1540.

436 Ozbek, N., & Akman, S. (2016). Determination of Chlorine in Milk via Molecular
437   Absorption of SrCl Using High-Resolution Continuum Source Graphite Furnace
438   Atomic Absorption Spectrometry. *Journal of Agricultural and Food Chemistry,
439   64*, 5767-5772.

440 Peng, B., Wu, D., Lai, J., Xiao, H., & Li, P. (2012). Simultaneous determination of
441   halogens (F, Cl, Br, and I) in coal using pyrohydrolysis combined with ion
442   chromatography. *Fuel, 94*, 629-631.

443 Pereira, É. R., Kopp, J. F., Raab, A., Krupp, E. M., Menoyo, J. d. C., Carasek, E., Welz,
444   B., & Feldmann, J. (2016). Arsenic containing medium and long chain fatty
445   acids in marine fish oil identified as degradation products using reversed-phase
446   HPLC-ICP-MS/ ESI-MS. *Journal of Analytical Atomic Spectrometry, 31*, 1836–
447   1845.

448 Pereira, É. R., Rocha, L. M., Cadorim, H. R., Silva, V. D., Welz, B., Carasek, E., & de
449   Andrade, J. B. (2015). Determination of chlorine in coal via the SrCl molecule
450   using high-resolution graphite furnace molecular absorption spectrometry and
451   direct solid sample analysis. *Spectrochimica Acta Part B: Atomic Spectroscopy,
452   114*, 46-50.

453 Pereira, É. R., Welz, B., Lopez, A. H. D., de Gois, J. S., Caramori, G. F., Borges, D. L.
454   G., Carasek, E., & de Andrade, J. B. (2014). Strontium mono-chloride — A new
455   molecule for the determination of chlorine using high-resolution graphite
456   furnace molecular absorption spectrometry and direct solid sample analysis.
457   *Spectrochimica Acta Part B: Atomic Spectroscopy, 102*, 1-6.

458 Smith, F., McMurtrie, A., & Galbraith, H. (1977). Ion Chromatographic determination
459   of sulfur and chlorine using milligram and submilligram sample weights.
460   *Microchemical Journal, 22*, 45-49.

461 Welz, B. (2004). High-resolution continuum source AAS: the better way to perform
462   atomic absorption spectrometry. *Analytical and Bioanalytical Chemistry, 381*,
463   69-71.

464   Welz, B., Lepri, F. G., Araujo, R. G. O., Ferreira, S. L. C., Huang, M.-D., Okruss, M.,
465   & Becker-Ross, H. (2009). Determination of phosphorus, sulfur and the
466   halogens using high-temperature molecular absorption spectrometry in flames
467   and furnaces—A review. *Analytica Chimica Acta, 647*, 137-148.

468   Welz, B., Vale, M. G. R., Pereira, E. R., Castilho, I. N. B., & Dessuy, M. B. (2014).
469   Continuum source atomic absorption spectrometry: Past, present and future
470   aspects-a critical review. *Journal of the Brazilian Chemical Society, 25*, 799-
471   821.

472

473

474

Figure captions

Figure 1. Time-resolved absorbance spectrum of the SrCl molecule in the vicinity of the most sensitive line at 635.862 nm obtained from 10 µL of a solution (0.4 g of raw fish oil diluted to 2 mL with 1-propanol) pipetted onto the Zr-treated PIN-platform in the graphite tube and 10 µL of a 1% (m/v) Sr^{2+} solution (0.10 mg Sr^{2+}) as molecule-forming reagent.

Figure 2. (a) Pyrolysis and (b) vaporization curves for the SrCl molecule using (●) 10 µL of 0.2 g mL^{-1} of raw fish oil diluted in 1-propanol, and (■) 15 µL of aqueous standard solution of 1 mg L^{-1} Cl (15 ng Cl absolute).Vaporization temperature for the pyrolysis curve (a) has been 2200 ºC; pyrolysis temperature for the atomization curve (b) has been 1300 ºC; zirconium as permanent chemical modifier; 10 µL of 1% m/v Sr^{2+} solution (0.10 mg Sr^{2+}) as molecule-forming reagent.

Figure 3. Appearance of the three commercial fish oil samples used in this work.

Table 1. Temperature program used for the determination of Cl via the SrCl molecule in fish oil samples using HR-CS GF MAS; an argon gas flow-rate of 2.0 L min^{-1} was used in all stages, except in the vaporization stage, where it was turned off.

Stage	Temperature / °C	Ramp / °C s^{-1}	Hold time / s
Drying 1	110	10	25
Drying 2	120	10	20
Pyrolysis	1300	50	15
Vaporization	2100	3000	5
Cleaning	2400	1000	4

Table 2. Figures of merit for the determination of Cl in fish oil samples by HR-CS GF MAS.

Parameter	Chlorine
Absorption wavelength[1], nm	635.862
Limit of detection[2], ng	1.8
Limit of detection[3], $\mu g\ g^{-1}$	0.9
Limit of quantification[4], $\mu g\ g^{-1}$	3.0
Characteristic mass m_0[5], ng	0.32
Correlation coefficient, R^2	0.998
Linear working range, ng	3 - 80
Slope of calibration S, $s\ ng^{-1}$	0.0136

[1] integrated absorbance using three pixels (CP±1)

[2] calculated as 3 σ/S (n = 10)

[3] considering 0.4 g of sample diluted to 2 mL using 1-propanol

[4] calculated as 10 σ/S (n = 10), considering 0.4 g of sample diluted to 2 mL using 1-propanol

[5] calculated as 0.0044 s / S

Table 3. Chlorine determination in fish oil samples by HR-CS GF MAS and micro-coulometric titration. The values represent the mean of three measurements ± standard deviation (SD).

Sample	Concentration / µg g^{-1} ± SD		
	HR-CS GF MAS	Coulometric titration	p-value[1]
Raw	56.9 ± 2.8	55.2 ± 0.7	0.22
Bandolado	30.3 ± 0.5	28.1 ± 0.6	0.05
Crude	26.4 ± 0.5	25.7 ± 0.3	0.10
Sample A	11.5 ± 0.9	11.4 ± 0.9	0.90
Sample B	10.1 ± 0.8	11.5 ± 0.1	0.15
Waste fraction	31.1 ± 2.0	nd	-
Intermediate product	3.5 ± 0.2	nd	-
Commercial 1	17.2 ± 0.6	nd	-
Commercial 2	< LOQ[2]	nd	-
Commercial 3	< LOQ[2]	nd	-
BCR 181	nd	1400 ± 21.0[3]	0.43

nd = not determined

[1] based on a Student t-test at a 95% confidence level ($p > 0.05$)

[2] 3.0 µg g^{-1} calculated as 10 σ/S (n = 10), considering 0.4 g of sample diluted to 2 mL using 1-propanol
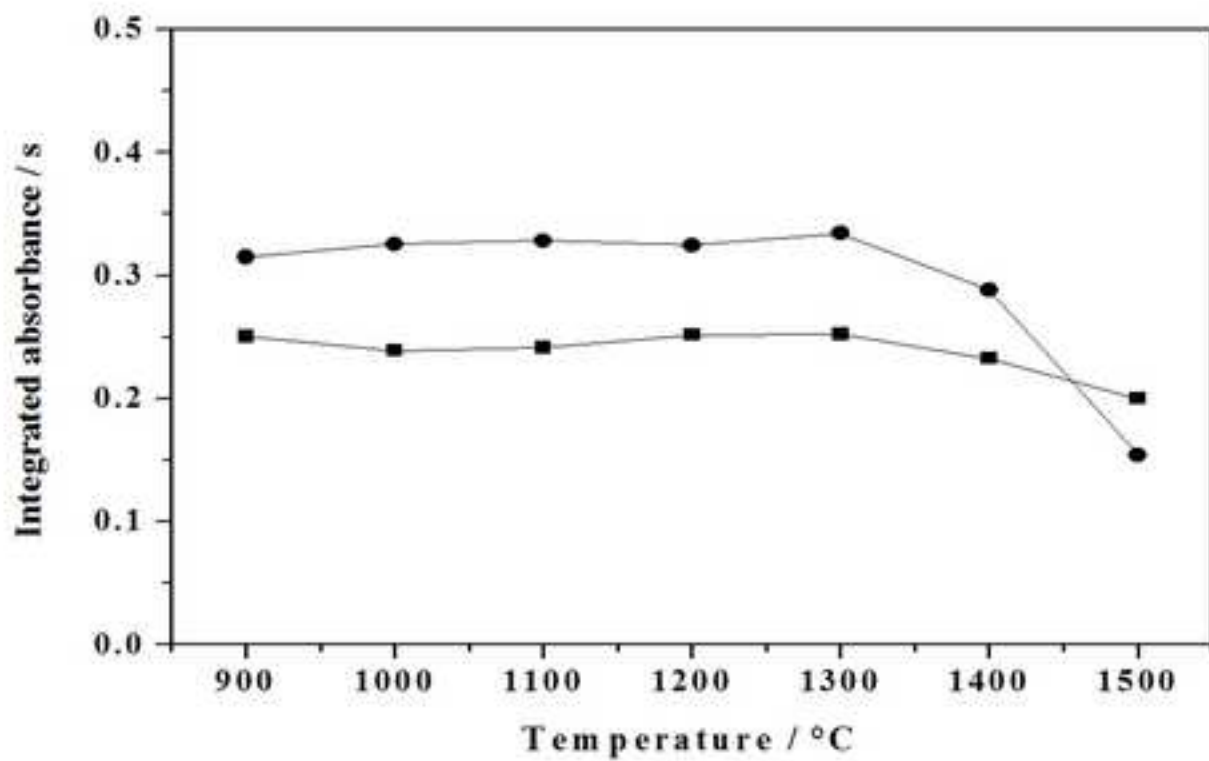
[3] certified value: 1380 ± 50 µg g^{-1}

Table 4. Content of omega-3 fatty acids in commercial fish oil capsules claimed on the labels; all values in mg g^{-1} normalized for 1 g of fish oil.

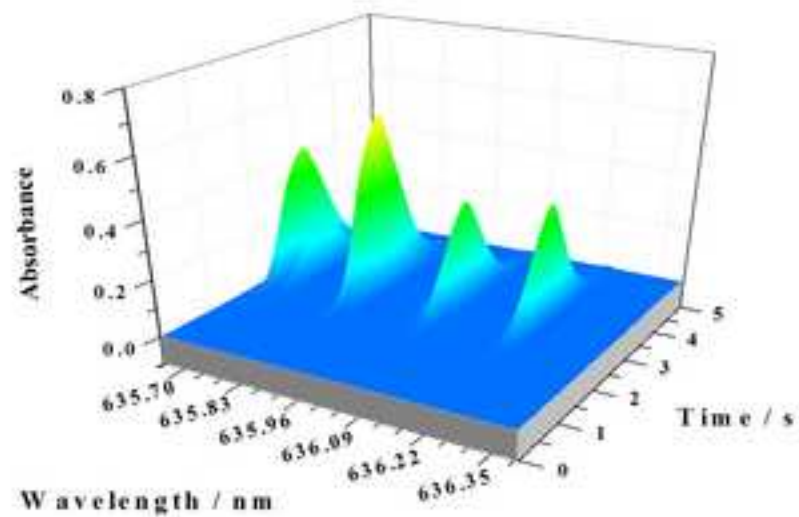| Sample No.	1	2	3
Denomination	Salmon oil, omega-3 concentrate	Omega-3 from salmon & fish oil	Omega-3 salmon oil
Omega-3	300	234	350
DHA	123	94	120
EPA	177	140	180
Σ DHA + EPA	300	234	300
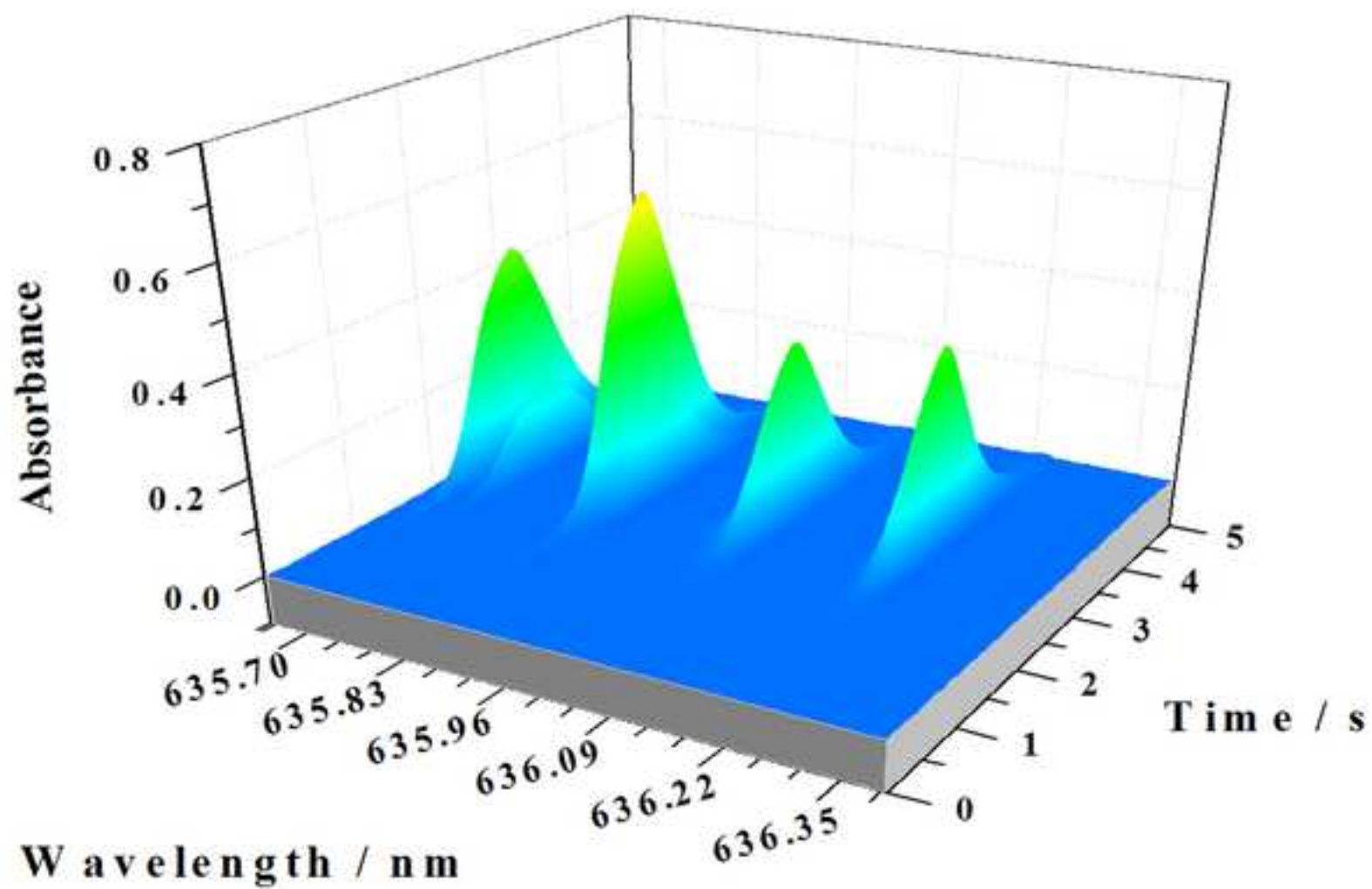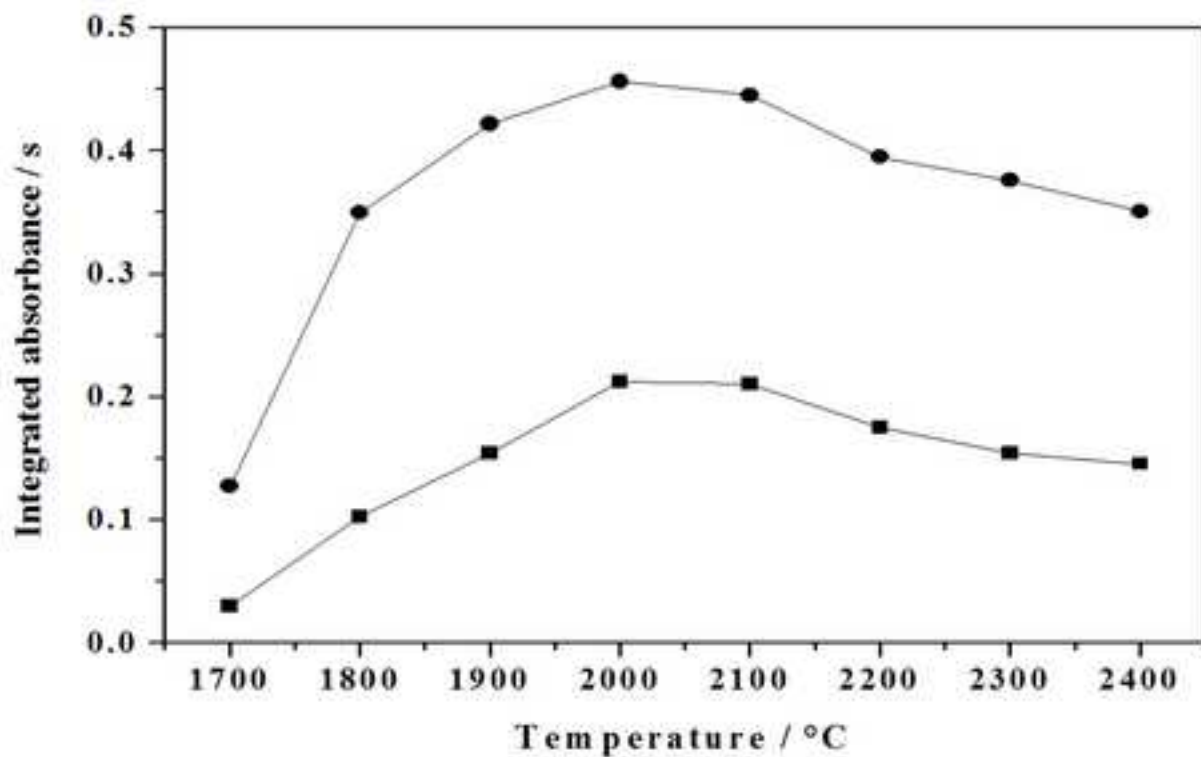
Highlights:

- Chlorine can be considered a proxy for persistent organic pollutants (POPs)
- High-resolution molecular absorption spectrometry was used for Cl determination
- Chlorine has been determined in fish oil for the production of Omega-3
- A method has been developed that needs only dilution as sample preparation
- A detection limit of 0.9 µg g^{-1} in diluted fish oil has been obtained.