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Pressurised liquid extraction and quantification of fat–oil in bread and derivatives products

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ABSTRACT

A pressurised liquid extraction (PLE) method for extraction and quantification of total fat and oil in bread and derivatives products has been proposed. Parameters implied in the extraction process; such us temperature, static time, number of extraction cycles, purge time and flush volume; have been optimised using a formal methodology based on statistical experimental design in order to obtain the best results. Moreover, this method has been validated using homemade bread elaborated in the laboratory which contained 9.64 g of olive oil in 100 g dry weight. The production and use of an "ad hoc" in-house reference material is just one of the most relevant aspects of this study. The uncertainty estimation has been carried out taking into account all the uncertainty components of the process and it was stated as 4.2%. Finally, the proposed method has been applied to six different Spanish bread derivatives products with different olive oil contents (5–20%) to determine the fat content.

1. Introduction

Nowadays the total lipid content of food products is an important issue in several studies. Lipids are a diverse group of biological substances made up primarily of non-polar compounds (acylglycerols, waxes and sterols) and more polar compounds (free fatty acids, phospholipids and sphingolipids). The complex nature of the total lipid composition of foods, from non-polar glycerides to polar phospholipids, means that the extraction with solvents has to be effective across a range of polarities. This is made more difficult because lipids bind to proteins (lipoproteins) and sugars (glycolipids) on cell membranes require a particular polar solvent to remove then [1]. Non-polar organic solvents, like hexane, are valid for neutral or simple lipids, which include fatty acid methyl esters, mono, di and triacylglycerols and unsaponifiable matter. Polar or complex lipids (such as phospholipids, glycolipids, lipoproteins, oxidised acylglycerols and free fatty acids) are extracted preferentially by polar solvents like methanol.

This ability of different solvent mixtures to dissolve different lipid classes has led to the concept of "total fat extraction". There are different ways to explain the total fat content in a sample: (i) substances extracted under the method conditions; (ii) total lipids including phospholipids; (iii) all the unchanged fatty acids from food; and (iv) the food lipids converted to triacylglycerols (net fat) and the sum of all lipids expressed as triacylglycerols [2].

The "ideal" extraction method should be quantitative, non-destructive, and low time- and solvent-consuming. Such a procedure is hard to imagine, considering the complexity of the lipids. Several methods have been developed for total lipid extraction which are based on the use of solvents or solvents combination, but the most common are a mixture of chloroform and methanol (Folch method, modified later by Bligh and Dyer), a mixture of diethyl ether and petroleum ether (Roese-Gottlieb or Mojonnier method), and n-hexane/2-propanol (3/2, v/v) (Hara and Radin method). Other solvent mixtures have been tested with different results. Detailed information on these topics can be found in any lipid or food analysis handbook [3,4].

To increase the extraction efficiency and allow a simultaneous treatment of numerous samples, several continuous extraction methodologies have been described. The method most used for solid food products is the *Soxhlet*. There have been several modifications in order to improve their lipid recovery efficiency, although the quantitative results of these different methods are similar [5,6]. Some automated or semi-automated fat analysers based on the Soxhlet device are commercially available such as the *Soxtec Extractor* [7] and *Ankom Fat Extractor* [8].

In addition, several other extraction techniques have been developed [4,9] such us, microwave-assisted extraction (MAE)

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[10], supercritical fluid extraction (SFE) [11], focused microwave-assisted Soxhlet extraction (FMASE) [12], dynamic ultrasound-assisted extraction (DUAE) [13] and pressurised liquid extraction (PLE, also named ASE, for accelerated solvent extraction) [14]. PLE is an extraction technique that combines elevated temperature and pressure with liquid solvents to achieve fast and efficient extraction. This high temperature causes an increase in the solubility of the analyte in the solvent, a fast diffusion and a better mass transference; consequently the solvent can seep easily through the matrix. This automation reduces solvent use and operating cost. Although the required instrumentation has a higher cost than the one from standard methods, this additional cost is worthy when the number of analysis is high and taking into account that the solvent cost is reduced. In conclusion PSE is faster and much more efficient than traditional methods. General characteristics of the main available extraction methodologies for solid samples which have been mentioned previously are discussed in Refs. [3,10].

Instrumental spectrometric techniques such as infrared spectroscopy (IR) [15] or nuclear magnetic resonance (NMR) [16] are being also used for the direct determination of fat in food, generating large amounts of data which provide information after chemometric treatment. These methods are quick but they have an important drawback since it is necessary to build a good mathematical model previously, they also show a poor precision.

Since the introduction of the first commercial PLE instrument a few years ago, PLE has proved to be a good alternative to replace other extraction methods. Recently, PLE has been exploited in different areas, including environmental pollutants [17], pharmaceuticals [18], biological materials [19,20] and foods [21]. For instance, in this last area, PLE has been applied to extract fat and oil from cereals [22,23] oilseeds [24], dairy products [25], fish tissues [26] and meat [27] using either pure solvents such as hexane and petroleum or mixtures such as hexane/acetone and hexane/dichloromethane/methanol. In addition, a formal strategy of statistical experimental design could be easily employed to deal with the simultaneous optimisation of all the variables but application examples of this methodology on PLE optimisation are scarcely found in scientific literature [28,29] and one-at-a-time variable optimisation is usually used.

Surprisingly, in spite of a great number of articles published where methods are described for the determination of total fat content in foods, it is only in a few cases that a proper validation study appears after the extraction method has been described [30,31]. A good single-laboratory validation practice requires the use of a representative certified reference material or similar, or the comparison with a recognised reliable analytical method. As a last resort, a well-organised method-performance collaborative interlaboratory study could be carried out. As a result, the method performance characteristics in terms of accuracy (trueness and precision), selectivity and range of application could be estimated [32]. In addition, both traceability [33] and uncertainty [34] of the results could be established.

This paper focuses on the development of an alternative analytical method, easy to apply, for the extraction of total fat/oil contents in bread and derivatives products using a pressurised solvent extractor, and subsequent quantification by gravimetric measurements. The variables of the method have been optimised using the statistical methodology of the design of experiments. The method has been validated properly using, as in-house reference material, bread elaborated in the laboratory with known olive oil content, and the expanded uncertainty of the results has been estimated. Finally, the proposed method has been applied to several commercially available samples of Spanish bread snacks containing olive oil as an ingredient.

2. Materials and methods

2.1. Solvents and reagents

All the solvents (analytical grade) used for extraction (hexane, 2-propanol, chloroform, methanol and ethanol) were supplied by PANREAC. Diatomaceous earth (supplied by Dionex) was used as inert solid. Deionised water was obtained from a purification system (Milli-Q; Millipore), and nitrogen (99.99%) was from Air Liquide.

2.2. Equipment and software

Extractions were performed on an Accelerated Solvent Extractor ASE 100 (Dionex), using 34 ml steel extraction cells. This equipment was validated for the extraction of non-bounded oil from solid samples by applying an internal procedure for quality assurance of pressurised liquid extractors. A calibrated three-figure analytical balance (Mettler Toledo PB303) was used for weight measurements. A Büchi RE-124 rotatory evaporator equipped with a vacuum pump V-700 (Büchi) was used to remove the remaining solvents after extraction. A household grinder (Taurus) was used for previous sample homogenisation and a household bread machine (Taurus) to elaborate bread containing a known olive oil content as homemade reference material for the validation process.

The Statgraphics Plus 5.1 software package [35] was used for statistical treatment and interpretation of collected data.

2.3. Sample extraction

Six characteristic spanish bread snacks (edible products made available in small sizes, which are attractive, appetizing, and ready to be eaten as such or in combination) named “Regañás”, “Picos”, “Palitos”, “Panecillos”, “Mini Tortas de Pan” and “Saladitos” which contained olive oil in different proportions (5–20%) were used as samples bread and derivatives products. They are all food products obtained from bread dough, comprising mainly flour, water, yeast, salt and edible fats, sugars, extracts and other conventional additives of this type. All samples were purchased from common markets.

Samples were grounded with a mixer to fine particles, until complete homogenisation. By decreasing the particle size, the surface area was increased and this results in an improvement on the efficiency of the extraction process. They were dried in a drying oven at 105 °C for 2 h. The samples were stored in opaque glass desiccators at room temperature until their analysis. Samples were weighed before and after the drying process in order to determine the moisture content (it was estimated that the analysed samples had an average moisture content of approximately 1.5%).

For the analysis, an amount of around 5 g of sample was exactly weighed with an approximation of 10 mg in a watch glass. Next the sample portion was placed in a mortar and mixed with 3 g of diatomaceous earth, and was then placed in the extraction cell, previously prepared with a cellulose filter to prevent clogging of the metal on the base of the extraction cell. The remaining dead volume was filled with diatomaceous earth (approximately 2 g). The prepared cell was placed on the equipment support and was then extracted into a 100 ml collection bottle. Each sample was analysed three times. The solvent extraction used was mixture hexane:isopropanol, 3:2 (v/v) (see Section 3.1). The operating pressure was 1500 psi.

For total fat yield data, the fat extract with the solvent was transferred into a 100-ml glass flask and the entire solvent was distilled in a rotary evaporator. The fat extract was then kept in a drying oven for 30 min at 60 °C to stabilisation of the weight and finally

the extracted mass was measured on an analytical balance at room temperature.

2.4. Production of an in-house reference material

Homemade bread was used as reference material to validate the proposed method. For production of this in-house reference material with a well-known amount of olive oil was used a commercial bread machine (TAURUS, My Bread). All the ingredients were carefully weighted with approximation 0.01 g before adding to the dough. The ingredients were water (300 g), sugar (5 g), salt (1.5 g), flour (450 g), yeast (3 g) and olive oil (76 g). The bread was weighed after baking and cooling and then the percentage of olive oil was calculated taking into account the amount of olive oil added firstly. After making this bread with olive oil, bread with the same amounts of ingredients, but without olive oil, was made in order to use as blank correction. The homemade bread elaborated in the laboratory contained a well-known amount of 9.64 g of olive oil in 100 g dry weight, that was added previously to the raw dough before baking and consequently it could be considered as in-house reference material. In a parallel way, it was elaborated bread that did not contain added olive oil for which it was obtained a fat content of 0.39 g/100 g dry weight. This blank correction was applied to the values of obtained fat content in the bread reference material.

This homemade bread could be used as representative material for any bread and derivative products since it had the same characteristics and ingredients. Moreover, there were not oil losses during bread preparation or baking due to an exhaustive weight control, which was carried out before and after the baking process.

3. Results and discussion

3.1. Optimisation of the extraction process

The proper application of a PLE method requires the optimisation of an instrumental variable set such as temperature, pressure, heating time, extraction time, number of extraction cycles, flush volume and purge time. In addition, these variables have to be optimised in conjunction with other analytical variables like the amount of sample, sample particle size, extraction solvent or hydrolysis conditions (when necessary).

Optimisation of the extraction process begins, generally, with the selection of an appropriate extraction solvent. Different experiences, based on what we found on bibliography for bread and derivatives products [20], were carried out. Different solvents and solvents mixtures were tested: chloroformo:methanol 2:1 (v/v) [36], ethanol [37], and hexane:isopropanol 3:2 (v/v) [38]. With the mixture chloroform: metanol 2:1, it was extracted a total fat content of 12.97% for a sample which had 15% of total fat. Extractions with ethanol gave incorrect results; they were greater that the nominal fat content, maybe either because of different compounds were extracted from the matrix or a wrong value on the label. The mixture hexane:isopropanol 3:2 (v/v), recommended by the extractor supplier, was found to be more effective than the others and it was chosen as the extraction solvent.

The ASE 100 extractor can use extraction cells of 10, 34, 66 and 100 ml and a collection bottle of 250 ml. The 34 ml cell was selected because it is suitable for 5 g of sample containing 5–20% fat. Next, the main variables implied in the pressurised liquid extraction process (temperature, static time of extraction, flush volume, purge time and number of extraction cycles) were optimised by applying a methodology of statistical design of experiments in two steps: (i) screening of significant variables, and (ii) establishment of the selected variable optimum values. The operating pressure was not optimised because the ASE 100 only operates at 1500 psi (100 bar). For the optimisation process, due to all the bread and derivatives products used in this work had similar composition, one of the snacks, "regañás", was used as a test sample to optimise the extraction process.

Table 1
Variable experimental domain to be optimised.

Variables	Code		
	−1	0	+1
A = Temperature (°C)	100	125	150
B = Number extraction cycles	1	3	5
C = Static time (min)	3	5	7
D = Purge time (s)	50	100	150
E = Flush volume (ml)	20	60	100
F = Dummy #1	–	–	–
G = Dummy #2	–	–	–

For the variable screening analysis, a 2-level saturated fractional factorial design for 5 variables (2^{7-4}) resulting in an 8-run experimental matrix was applied; the experimental domain (Table 1) of the investigated variables was determined based on information from the equipment supplier.

The design matrix and the results of each experimental run, where the response variable is the measured fat content (F) expressed in grams of fat per 100 g of dried sample (g/100 g dry weight), and the effect of each variable, are presented in Table 2. The experimental runs were carried out randomly to avoid occasional effects on the variables and all the experiences were carried out carefully by qualified personnel.

Two "dummy variables" were added to complete the mathematical structure of the design matrix, although they do not have any physical-chemistry meaning and, therefore, they do not produce any effect in the experimental response.

The effect of the variables and their importance are graphically evaluated by a pareto-chart of standardised effects (Fig. 1). A value of ±0.4 g/100 g dry weight was selected by us as the in-house significance threshold based on previous experiences and on acceptable errors for fat extraction (<5% experimental mean value). Only two variables, temperature and purge time, show a significant effect on the extracted fat content. The temperature shows the highest positive effect (+0.51 g/100 g dry weight for each 25 °C temperature change) which implies that the higher the temperature, the higher the obtained fat content. The effect of extraction temperature could be explained by the increase of the diffusion coefficient in the liquid solvent into the solid matrix while the extraction temperature increases, favouring the kinetics of desorption of the compounds from the matrix [39]. Also, higher temperatures enhance the vapour pressure (volatility) of extract compounds favouring the extractions from the vegetable porous matrix. Purge time shows the lowest negative effect (−0.39 g/100 g dry weight for each 50 s inter-

Table 2
Experimental matrix and measured fat content for each experimental run, and estimated effects for each variable. The fat content and effects are expressed in grams of extracted fat per 100 g of dried sample (g/100 g dry weight).

Run	Variables							Measured fat content
	A	*B*	*C*	*D*	*E*	*F*	*G*	
1	+1	+1	+1	−1	−1	−1	+1	10.51
2	−1	+1	+1	+1	+1	−1	−1	9.16
3	+1	−1	+1	+1	−1	+1	−1	9.78
4	−1	−1	+1	−1	+1	+1	+1	9.50
5	+1	+1	−1	−1	+1	+1	−1	9.96
6	−1	+1	−1	+1	−1	+1	+1	9.54
7	+1	−1	−1	+1	+1	−1	+1	9.90
8	−1	−1	−1	−1	−1	−1	−1	9.92
Mean								9.78
Effects	0.51	−0.09	−0.31	−0.39	0.02	−0.18	0.16	

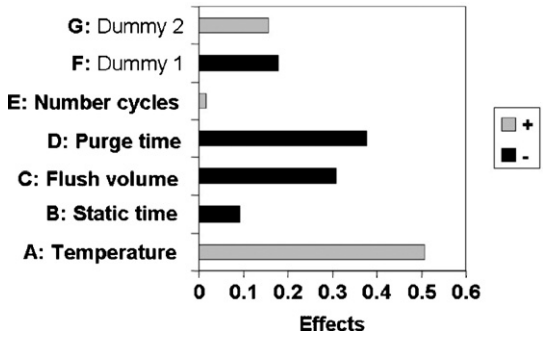

Fig. 1. "Pareto-chart" of standardised effects. The black bars represent the variables that produce negative effects on the responses (g/100 g non-dried sample) and the grey bars those variables that produce positive effects.

val) which implies that decreasing the purge time leads to a higher obtained fat content. This effect could be explained by the fact that during the purge process, some amount of fat extract could be lost due to the high pressure which it takes place. Flush volume, static time, and number of extraction cycles do not show significant influence on the extraction process in the experimental domain studied. In addition, significant interactions between variables are not observed.

Next the two significant variables were studied in order to find the optimal value for each. First the area around the central point of the experimental domain was studied to determine the direction of highest rise in the experimental response in order to continue the experimental study in this direction. The greatest measured fat content was found for minimum values of purge time and maximum values of temperature. Thus the optimum values for purge time and temperature were located at 100 s and 175 °C, respectively. Fig. 2 illustrates the optimisation strategy followed and the obtained responses in each case.

Because of the effects of the remaining variables (static time, flush volume and number of extraction cycles) were not significant, the operating values were chosen according to the more favourable operation for the process. Static time (5 min) was selected according to the central point of the experimental domain (see Table 1), flush volume (40%) was chosen because at this value the extraction efficiency was not affected and the solvent cost was minimal, at last the number of extraction cycles (1 cycle) was selected because the fat content did not differ too much and the extraction time was less.

Finally, the optimised conditions for extraction were a temperature of 175 °C, heating period of 5 min, extraction (static) time of

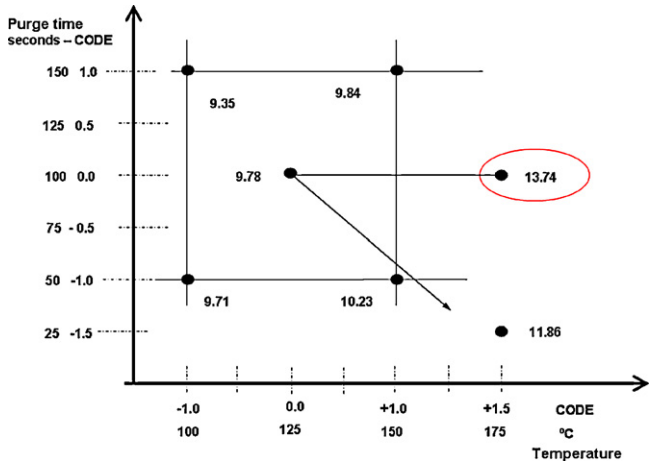

Fig. 2. Experimental strategy followed in the simultaneous optimisation of both extraction temperature and purge time.

5 min with one static extraction cycle per sample, flush volume of 40% and purge time 100 s. The overall time required for the extraction was 12 min.

3.2. Method validation

The validation procedure was carried out by applying the analytical method, at the operation conditions previously optimised and using homemade bread produced in the laboratory. The average net recovery obtained, once the blank correction is applied, after ten extractions from different portions of the reference bread was 9.57 g/100 g dry weight with a standard deviation of 0.211 g/100 g dry weight.

The precision and trueness of the method, expressed as relative standard deviation (RSD) and recovery ($\Re$) in percentage, were 2.11% and 99.27%, respectively. The estimated precision is similar to other analytical methods of determination of total fat in food by extraction [30] but significantly better than the instrumental techniques of direct determination. It is observed that there is no significant bias because $|100 - \Re| \ll$ RSD and so the trueness of the analytical method is satisfactory. In addition, the use of a calibrated balance and a qualified extractor jointly with the high-purity solvents and the room temperature control, assure the traceability and the comparability of the results.

An estimation of the limit of quantification (LOQ) of the method was also calculated from 10 times the standard deviation. The analytical method is suitable for analysis of bread and derivatives products with a total fat and oil content no less than 2 g/100 g dry weight.

3.3. Uncertainty estimation

For the uncertainty estimation, a parameter that characterises the dispersion of the results obtained from the analytical method, the following steps are followed [34]:

3.3.1. Specifying the measurand

The measurand is the total fat content, F, expressed in grams of fat per 100 g of non-dried sample (g/100 g non-dry weight).

3.3.2. Modelling the measurement

Express mathematically the relationship between the measurand and all of the input quantities upon which the measurand depends. The equation for quantifying the total fat content:

$$F = \left[\left(\frac{m_1 - m_0}{M_1 - M_0}\right)_{\text{reference sample}} - \left(\frac{m_1 - m_0}{M_1 - M_0}\right)_{\text{blank sample}}\right] \times 100 = \left[\left(\frac{\Delta m}{\Delta M}\right)_{\text{reference sample}} - \left(\frac{\Delta m}{\Delta M}\right)_{\text{blank sample}}\right] \times 100$$

where: m_0 is the weight, in grams, of empty flask used for the extracted fat collection; m_1 is the weight, in grams, of collection flask with the extract; M_0 is the weight, in grams, of the empty watch glass used to weigh the sample; and M_1 is the weight, in grams, of the watch glass with the sample. The terms Δm and ΔM designate the measured mass of analyte (fat) and sample, respectively.

3.3.3. Quantifying the uncertainty components and their associated uncertainties

Estimate the value of each input quantity either by the statistical analysis of repeated observations or by other means such as taking the uncertainty of a reference standard from a calibration certificate. The obtained values constitute the standard uncertainty components, u_i. In Fig. 3 all of the possible uncertainty components

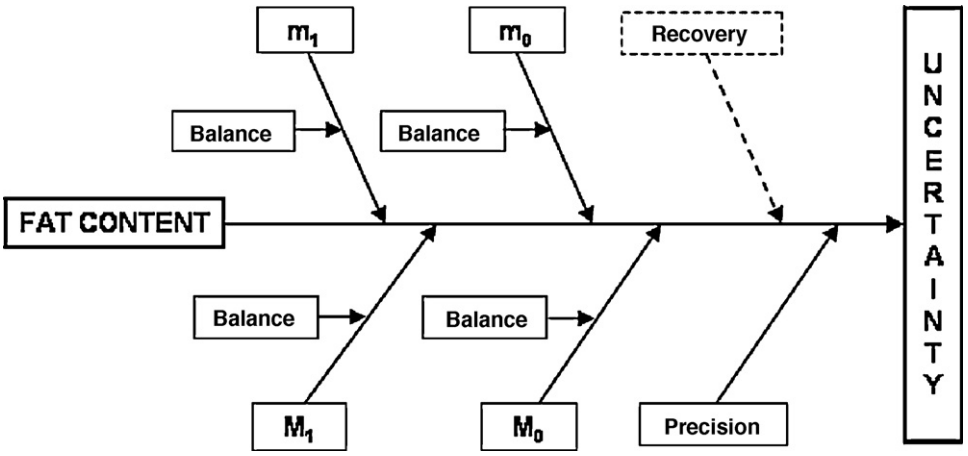

Fig. 3. Cause-effect diagram of uncertainty components in the method validation process. All the components are related to the weight and the balance. (The dashed-line represents the uncertainty component due to the recovery. In this uncertainty budget this component is not taken into account because the recovery has a non-significant value.)

in the extraction process are shown by means of a cause-effect diagram. It is observed that all of them are related to the weight.

3.3.4. Combining the components

Calculate the combined standard uncertainty of the measurement result, $u(F)$, from the standard uncertainty components. The expression for uncertainty estimation is:

$$\frac{u^2(F)}{F^2} = \left(\frac{u^2(m_1) + u^2(m_0)}{(m_1 - m_0)^2} + \frac{u^2(M_1) + u^2(M_0)}{(M_1 - M_0)^2} \right) \times 2 + \mathrm{RSD}^2_{\mathrm{precision}}$$

or

$$\frac{u^2(F)}{F^2} = \left(\frac{u^2(\Delta m)}{\Delta m^2} + \frac{u^2(\Delta M)}{\Delta M^2} \right) \times 2 + \mathrm{RSD}^2_{\mathrm{precision}}$$

(Factor 2 is introduced because the determination is based on two complete analytical tests, one on the blank and another one on the sample.)

3.3.5. Simplifying the expression

In order to simplify the formula, the uncertainty of mass subtractions, whose measured mass values do not differ too much from each other, can be expressed as:

$$u^2(\Delta m) = u^2(m_1) + u^2(m_0) \approx 2u^2(m)$$

Reason why by including these terms in the initial equation of uncertainty calculation:

$$\frac{u^2(F)}{F^2} = 4 \times \left(\frac{u^2(m)}{\Delta m^2} + \frac{u^2(M)}{\Delta M^2} \right) + \mathrm{RSD}^2_{\mathrm{precision}}$$

The within-laboratory estimated uncertainty associated with each one of these mass measurements (<0.2%) is small in relation to the precision relative standard deviation (~2%) and so it can be considered negligible. Because of this the formula can be simplified still more:

$$\frac{u^2(F)}{F^2} \approx \mathrm{RSD}^2_{\mathrm{precision}} \Rightarrow \frac{u(F)}{F} = u_{\mathrm{rel}}(F) \approx \mathrm{RSD}_{\mathrm{precision}}$$

As the representative value of precision, RSD was given the obtained value in the method validation procedure. In conclusion, the established relative standard uncertainty of the method, $u(F)$, is 2.11%.

Table 3
Results obtained in the derivatives products of the bread analysis with their value of fat content provided by the manufacturer on the label and the measured values, with their expanded uncertainty ($k = 2$), when the proposed method extraction is applied. The fat content values are expressed in g/100 g non-dried sample.

Samples[a]	Fat content on the label	Measured fat content ± uncertainty
"Regañás"	15.8	14.2 ± 0.6
"Picos"	5.3	7.3[b] ± 0.3
"Palitos"	12.0	11.7 ± 0.5
"Panecillos"	21.0	22.5 ± 1.0
"Mini Tortas de Pan"	14.0	14.6 ± 0.6
"Saladitos"	19.8	17.6 ± 0.8

[a] Derivatives products of the bread, obtained from a bread dough, comprising mainly flour, water, yeast, salt and edible fats, sugars, extracts and other conventional additives of this type.
[b] This value shows a greater difference according to the fat content on the label fat content. It might be due to a mistake on the label provided by the manufacturer.

3.3.6. Calculate the expanded uncertainty

The expanded uncertainty, $U(F)$, is calculated by multiplying the combined standard uncertainty with the coverage factor k ($k = 2$ when a coverage probability of 95% is considered). So, the relative expanded uncertainty for the analytical method is stated as 4.2%.

3.4. Application to real samples

The developed validated method has been applied to the determination of the fat content in six characteristic Spanish snacks, quoted in Section 2.3. The obtained value for each sample, expressed in grams of fat per 100 g of non-dried sample, and their uncertainty are shown in Table 3. In addition, the value that appeared on the label provided by the manufacturer is shown.

As it can be seen the values of measured fat content are approximately similar to the values provided by the manufacturer on the label (except for one of them, it might be due to a mistake on the label provided by the manufacturer). These values from the labels have not been used to validate the method. They were only used as reference values in order to compare with our values. As it is shown in Table 3, for all the products tested, both values, the values on the label, and the found values, were quite consistent, which proves the applicability of the proposed method for the intended purpose.

4. Conclusions

As it can be seen from the present communication, pressurised liquid extraction technique together with the proposed gravimetric analytical method can be successfully applied for the extraction and quantification of total fat and oil from different bread and derivatives products. Also, the proposed method could be applied for food routine laboratories to quantify fat. The extractor was qualified properly following an internal procedure before being used. A mixture of hexane:isopropanol 3:2 (v/v) was selected as solvent extraction and the remaining variables were optimised with an statistical methodology. The extraction method has been validated. Moreover, the expanded uncertainty for the analytical method has been calculated with a value of 4.1% and the limit of quantification (LOQ) is established to be 2 g/100 g dry weight. Finally, the method has been tested on six commercial samples. The performance characteristics of the proposed analytical method reported here and the obtained results permit to conclude that PLE is a suitable technique for the quantification of total fat on bread and derivatives products. Furthermore, the possibility of coupling PLE with other steps in the analytical process is one of the most interesting aspects of this methodology.

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