Evaluation of chemical indices for the identification of incubator-reject eggs in egg products

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Abstract

The use of incubator-reject eggs (IRE) is not allowed for the preparation of egg products. However, some producers fraudulently use them for whole egg products manufacture. The aim of this research was to study the efficiency of European legislative indices (β-hydroxybutyric acid and lactic acid), uracil, furosine and organic acids for IRE detection in egg products. The results confirm the possible illegal IRE presence in egg products by selection of IRE eggs through candling and/or dilution with sound eggs. A revision of the European legislation thresholds, lowering the limits from 10 to 6 mg/kg dm and from 1000 to 600 mg/kg dm, respectively, is urgently needed. Furthermore, uracil concentrations ≥0.9 mg/kg dm should be considered a warning signal, so uracil is suggested as a future additional legal parameter. A complementing high resolution mass spectrometry screening test also identified β-hydroxybutyric acid, lactic acid and uracil as discriminative markers of IRE presence.

Keywords: β-hydroxybutyric acid; High resolution mass spectrometry; Incubation; Lactic acid; Non-target screening; Organic acids; Pasteurized whole-egg; Uracil

1 Introduction

Pasteurized whole egg, the most commonly marketed egg product (Lechevalier et al., 2017), is used by the food industry for the manufacture of bakery products (Cepeda-Vázquez, Camel, Blumenthal, & Rega, 2019) ice cream, fresh and dried pasta (Alamprese, Rossi, Casiraghi, Hidalgo, & Rauzzino, 2004; Verardo, Riciputi, Messia, Marconi, & Caboni, 2017). Egg products can be prepared with grade A (fresh) as well as with grade B (second quality) hen shell eggs; broken and incubator eggs are not allowed for human consumption (EU, 2008). However, some producers fraudulently use incubator-reject eggs (IRE) in the preparation of egg products even if they are considered unfit for human food (USDA, 2018). Whole egg is particularly exposed to this type of sophistication, as egg white and yolk are not separated. The bakery products are the foods most at risk because their production necessitates high temperatures and other ingredients, which can mask the low quality of egg product.

The IRE are unfertilized eggs or eggs fertilized but with an embryo dead in the first days of incubation; they are rejected at candling and can represent 5.0–13.8% (Damaziak, Pawęska, Gozdowski, & Niemiec, 2018) or 5.4–8.2% (Londero et al., 2015) of the total incubated eggs. Candling is generally performed on shell eggs after 10 days (egg production for vaccines manufacturing; Tseng et al., 2019) or after 18 days (chick production for laying hens or meat production) of incubation at 37–38 °C and 50–60% relative humidity under adequate ventilation and rotation (Damaziak et al., 2018; Moreno et al., 2018; Zakaria et al., 2005).

The European legislation (EU, 2005) establishes that egg products must not contain *Salmonella* and restricts the presence of *Enterobacteriaceae* to maximum 100 cfu/g. The legislation (EU, 2004) also limits the content of β-hydroxybutyric acid (β-OHbutyric acid), an index of IRE presence, to 10 mg/kg dry matter, and of lactic acid, a chemical index of hygienic quality of the raw material, to 1000 mg/kg dry matter. The β-OHbutyric acid is developed only by embryonic metabolism and not by microbial activity (Salwin, Staruszkiewicz, & Bond, 1972), thus is absent in non-fertilized incubated eggs (Robinson, Barnes, & Taylor, 1975). In eggs with live embryo, β-OHbutyric acid increases during the first 14 days of incubation, then decreases; in eggs where embryo growth stopped in the very first days, steadily increases during incubation (Salwin et al., 1972). Lactic acid is an index of both microbial contamination and

embryonic development (Cattaneo & Balzaretti, 1989). Besides the chemical legislation parameters, other metabolites could be interesting for the identification of IRE. Uracil, absent in sound whole eggs and formed because of the enzymatic hydrolysis of uridine by microorganisms, was suggested as a marker for assessing raw material hygienic quality in pasteurized egg products (Hidalgo, Franzetti, Rossi, & Pompei, 2008; Hidalgo, Rossi, Pompei, & Casiraghi, 2004) and fresh egg pasta (Alamprese et al., 2004). Furosine, formerly proposed as an egg freshness marker (Hidalgo, Rossi, & Pompei, 1995, 2006) could be present in IRE because the incubation conditions may favour the development of the first steps of Maillard reaction in eggs. Additionally, non-targeted approaches proposed for the characterisation of egg products spoilage (Coat et al., 2018) could also be considered.

The objective of this research was to determine the effectiveness of the legislative control indices of egg products (β-OHbutyric and lactic acids) as well as of uracil, furosine and other potential organic acids in detecting the fraudulent use of incubator-reject eggs in whole egg products. For this aim, the natural variability of these indices in IRE from an industrial incubator was investigated. In addition, the indices sensitivity was evaluated in whole egg samples prepared from IRE selected by candling or after their dilution with sound shell eggs, as in hypothetical illegal practices. Furthermore, a “non-target screening” high resolution mass spectrometry study was performed to verify which markers were the most discriminant for incubated eggs.

2 Materials and methods

2.1 Materials

In preliminary tests IRE from an industrial hatchery were analyzed:

- five batches of shell eggs, collected after 18 days of incubation; each lot consisted of 60 eggs
- five batches of mixes from broken eggs, obtained after 18 days of incubation, collected and frozen in the production plant, stored at −20 °C until analysis.

For the evaluation of natural variability and influence of candling, the following samples were used:

- twelve batches of eggs discarded after 10 or 18 days of incubation and laid by 34, 44 or 76 weeks old hens of the Novagen White or Novagen Brown breed, and two batches of not-incubated, fertilized eggs. Each lot contained 90–120 eggs. The egg batches were supplied by a second industrial hatchery, which produces incubated eggs for vaccines manufacturing (after 10 days) and for laying hens breeding (after 18 days). In vaccines production, eggs with live embryo but with weak or poorly developed amniotic sac are also discarded.
- a batch of fresh eggs of category A from the commercial market.

For the evaluation of the influence of dilution with sound fresh eggs (category A) were used:

- six batches of IRE collected after 18 days of incubation from the second industrial hatchery.

All egg batches were stored at 4 °C until sample preparation and analysis.

This research was carried out on incubator-reject eggs from industrial hatcheries, therefore the consent of an Ethical Commission was not necessary.

2.1.1 Sample preparation

Groups of six eggs were manually shelled and mixed with a Stomacher® 400 Circulator homogenizer (Seward, West Sussex, UK) for 30 s at 230 rpm. Subsequently, the different homogenates were pooled into a beaker and further mixed for 15 s with a Braun MQ100 Soup immersion stirrer (Warsaw, Poland).

2.1.2 Selection of IRE by candling at lab

In a preliminary test, we found that candling selection in industrial hatcheries did not totally remove eggs with middle or late embryo mortality; thus the batches 7, 8, 11 and 12 used for the evaluation of natural variability were further screened by candling in our lab and divided in two groups: selected eggs (absence of dark areas) and discarded eggs (dark or opaque interior). Candling is the visual control of the egg in darkness against a light. This operation allows to observe the transparency of the egg (uniform pink color for fresh eggs, dark and opaque color for non-fresh or incubated with embryonic development eggs) and to visualize the yolk (a diffused shadow; in contrast, embryonic development or age increase give a clearer shadow).

2.1.3 Dilutions

Whole egg from commercial grade A eggs was diluted with different percentages of IRE of two mixes (A and B) from different batches:

- Mix A: whole egg with 0, 2, 5, 10, 100% IRE
- Mix B: whole egg with 0, 5, 10, 15, 18, 100% IRE.

2.2 Total mesophilic aerobic bacterial count

The total mesophilic aerobic bacterial count was determined following AOAC method 966.23 (AOAC International, 1995a) in whole eggs from the five shell eggs batches of the preliminary trial.

2.3 Chemical analyses

Dry matter content (g/100 g) was determined following AOAC method no. 925.30 (AOAC International, 1995b). Protein content was calculated as total nitrogen multiplied by 6.25 and expressed as grams of protein per 100 g of product. Total nitrogen analysis was performed using Kjeldhal method n. 925.31 (AOAC International, 1995b).

β-OHbutyric acid was evaluated using the enzymatic kit Boehringer Mannheim/R-Biopharm (Darmstadt, Germany). Lactic acid, uracil, pyroglutamic acid, formic acid, uridine and uric acid analyses were performed by HPLC following Hidalgo et al. (2008). Sample preparation consisted of the deproteinization of 2 g whole egg in the presence of 2.8 mL HPLC water, 4.8 mL 6% perchloric acid, and 0.6 mL acetronitrile. After agitation using a vortex (Velp Scientifica, Italy) for 30 s and an orbital stirrer (PTR-35, Grant-bio, England) for 30 min the sample was centrifuged for 10 min at 12,000 *g* (10360 rpm) using a Centrikon T-42K centrifuge (Kontron Instruments, Milton Keynes, Buckinghamshire, UK). The supernatant was filtered through a 0.22 µm PTFE membrane (Sigma Aldrich Srl, Milan, Italy). A volume of 20 µL of filtered solution was injected in the HPLC system under the following operating conditions: column Aminex HPX87H, 300 × 7.8 mm (Bio-Rad Laboratories, Hercules, CA); column temperatures, 45 °C; guard column, Cation H cartridge (Bio-Rad Laboratories, Hercules, CA); mobile phase, 0.01 N sulfuric acid; flow rate, 0.6 mL/min; pump, Waters 510 (Millipore, Milford, MA). Uracil and uridine were detected at 260 nm; pyroglutamic and formic acids were detected at 210 nm, while uric acid was detected at 284 nm using a Millipore Waters 996 series photodiode array detector (Milford, MA) controlled by the software Millenium 32 Chromatography Manager (Waters Chromatography Division, Millipore, Milford, MA). The wavelength range used was 200–290 nm. Lactic acid was detected using a refractive index detector (model 1037A, Hewlett-Packard, Geneva, Switzerland) connected to a D-2500 chromato-integrator (Merck Hitachi, Tokyo, Japan). For peak quantification, calibration curves were built using 7 concentrations (between 4.0 and 1500 mg/L) of lactic acid standard (Supelco, Bellefonte, PA), 6 concentrations (between 0.2 and 2.4 mg/L) of uracil standard (Merck, Darmstadt, Germany), 5 concentrations (between 1.6 and 62 mg/L) of pyroglutamic acid standard (Sigma Chemical Company, St. Louis, MO), 8 concentrations (between 4.0 and 1000 mg/L) of formic acid standard (BDH® VWR Chemicals, France), 5 concentrations (between 1.6 and 62.0 mg/L) of uridine standard (Sigma Chemical Company, St. Louis, MO), and 5 concentrations (between 1.0 and 10 mg/L) of uric acid standard (BDH Laboratory Supplies, Poole, England), all in water. Based on the calibration curve, the limit of detection was calculated as the intercept value of the regression line plus 3 times the standard error of the estimate (Miller & Miller, 1988).

Furosine content was determined by HPLC following the method described by Hidalgo et al. (1995); 500 mg of sample were hydrolysed with 8 mL of 8 N HCl under nitrogen at 110 °C for 23 h and purified by solid-phase extraction with a C18 cartridge (Sep-pak, Millipore, Ballerica, MA, USA) and injected in a HPLC apparatus consisting of two 510 HPLC pumps, a 680 automated gradient controller, and a Waters 996 PDA detector (Waters, Milford, MA) controlled by the software Millenium 32. Furosine was quantified using furosine dihydrochloride (NeoMPS, PolyPeptide Laboratories, Strasbourg, France) as external standard. The results are expressed as milligrams of furosine/100 g protein. A calibration curve was built, using eleven different concentrations (between 0.55 and 55.6 µmol/L) of hydrated furosine 2 HCl (Neosystem Laboratoire, Strasbourg, France) in 3 N HCl.

The results are the average of duplicate measurements except for furosine analysis, performed in triplicate in the preliminary trial.

The “non-target” high resolution mass spectrometry (HRMS) study was performed using a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Inc., Waltham, MA) coupled with a benchtop Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) on the twelve IRE batches used for the evaluation of natural variability. The analytical conditions for both sample preparation and instrumental analysis were as described by Cavanna, Catellani, Dall’Asta, and Suman (2018).

2.4 Statistical analysis

To evaluate the differences between batches, the data were processed using one-way analysis of variance (ANOVA); *t*-test was instead used for two-means comparisons. To study the natural variability of IRE, a multifactor ANOVA was performed considering incubation time, hen breed, and hen age as factors. When significant differences were found ($p \leq 0.05$), the least significant difference (LSD) test was performed with the Fisher method at 95% significance level. ANOVA, *t*-test and LSD were calculated using the statistical program STATGRAPHICS® Centurion. Mean values, standard error and coefficient of variation were calculated using the Excel program (Microsoft® Office Excel 2016).

3 Results and discussion

3.1 Calibration curves

Calibration curves for all the compounds were linear ($r^2 = 0.9997$; $p < 0.001$) in the concentration ranges considered. The detection limits in the standard solution for lactic acid, uracil, pyroglutamic acid, formic acid, uridine, and uric acid were 2.82, 0.05, 1.49, 1.61, 0.59, and 0.04 mg/L, respectively, corresponding to 45.1, 0.84, 23.9, 25.8, 9.5, and 0.8 mg/kg dm in whole egg, considering a mean dry matter content of 24 g/100 g. The detection limit of furosine was 0.21 µg/L, corresponding to 4.6 mg/100 g of protein in whole egg, considering a mean protein content of 11.6 g/100 g.

3.2 Preliminary trial

Table 1 shows the average content of β-hydroxybutyric acid, lactic acid, uracil and furosine in five batches of incubator-reject shell eggs and five batches of whole egg mixes obtained from IRE in the industrial hatchery. The β-OHbutyric acid is an index exclusively linked to embryo development. The results show that all lots have β-OHbutyric acid levels well above the legal limit (10 mg/kg dm), ranging from 21.5 to 136.5 mg/kg dm in shell eggs and from 163.6 to 542.7 mg/kg dm in mixes. The variability within each group, expressed as coefficient of variation, is very high (56% and 48%, respectively) and can be attributed to the simultaneous presence, in different proportions, of unfertilized eggs and eggs with dead embryo. This last group is the main contributor to the increase of β-OHbutyric acid content during incubation (Salwin et al., 1972).

Table 1 Total bacterial count (TBC; cfu/g), ANOVA (mean square values and significance), mean values, and LSD test for β-OHbutyric acid, lactic acid, uracil (mg/kg dm) and furosine (mg/100 g protein) contents in whole egg from five lots of incubator-reject shell eggs and five lots of incubator-reject broken eggs (mix). The *t-test* for comparison of the two types of discarded products is also presented.

alt-text: Table 1

Factor	TBC	df	β-OHbutyric	Lactic	Uracil	df	Furosine
Lot		9	47449.5***	4457960***	2190.49***	9	345.68***
Error		10	763.3	24799	0.17	20	8.76
Lot							
Shell eggs 1	10		136.5^{d}	2324.7^{e}	nd^{i}		72.1^{a}
Shell eggs 2	<10		21.5^{f}	2362.3^{e}	9.1^{g}		74.2^{a}
Shell eggs 3	<10		130.3^{de}	4647.2^{c}	78.2^{b}		56.2^{c}
Shell eggs 4	<10		74.5^{ef}	3312.3^{d}	1.2^{h}		62.0^{b}
Shell eggs 5	<10		67.1^{f}	3642.6^{d}	1.8^{h}		73.0^{a}
Mix 1			163.6^{d}	4860.1^{bc}	93.0^{a}		49.6^{d}
Mix 2			258.3^{c}	3662.2^{d}	43.9^{e}		75.3^{a}
Mix 3			327.7^{b}	5126.1^{b}	27.2^{f}		51.8^{cd}
Mix 4			542.7^{a}	7390.8^{a}	45.9^{d}		49.8^{d}
Mix 5			232.7^{c}	4526.2^{c}	50.2^{c}		53.0^{cd}
				Means and *t-test* results			
Shell eggs			86.0	3257.8	18.0		67.2**
Mix			305.0***	5113.1**	52.0*		55.9

nd, lower than the detection limit, ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, different letters within a column indicate significant differences (LSD test, p ≤ 0.05).

Lactic acid and uracil are indices of microbial contamination. Uracil is linked to lactic bacteria contamination (Hidalgo et al., 2008); lactic acid formation is connected to embryo metabolism, reaches a peak around the fifth day of incubation and then decreases to the initial values (Miraglia, 1989). The lactic acid concentrations largely exceed the legal limit for egg products (1000 mg/kg dm), because in eggs they ranged between 2324.7 and 4647.2 mg/kg dm, and in mixes between 3662.2 and 7390.8 mg/kg dm. The variation was lower (30% and 27%, respectively) than that of β-OHbutyric acid. Uracil is not a legal index, however its absence (below the detection limit) suggests good hygienic quality of the raw material used in liquid pasteurized egg products (Hidalgo et al., 2004, 2008). Hidalgo et al. (2004) registered detectable uracil concentrations in whole egg from non-incubated eggs only when the levels of contamination were superior to 10^5 cfu/g. It is interesting to note that even if the total bacterial count in the whole egg of the five IRE lots was very low (below or equal to 10 cfu/g; Table 1), two batches (lots 2 and 3; Table 1) had high quantities of uracil. This result could suggest that uracil is formed by both microbial contamination and embryo development. However, its development in IRE eggs is not always constant since the other three IRE batches presented very low or undetectable quantities (Table 1); as a consequence, the variability between lots was very high (196%). Uracil, on the other hand, was found in all the mixes, ranging between 27.2 and 93.0 mg/kg dm and with a variability of 47%, because the products are highly polluted by the shelling method. In fact, crushing puts in direct contact the interior of the egg with the shell, often for an indefinite period and at room temperature.

Furosine content ranged between 56.2 and 74.2 mg/100 g proteins in shell eggs and between 49.6 and 75.3 mg/100 g proteins in mixes; the variability was very low (11% and 20%, respectively), as a consequence of standard, common conditions of incubation for all lots. These results are higher than the whole egg levels (around 40 mg/100 g protein) reported by Hidalgo et al. (1995) during the storage at 20 °C of unfertilized eggs. Thus, furosine is not affected by fertilization and embryonic development, but incubation conditions favour its formation.

Comparing the overall mean values of the five IRE batches and the five batches of whole egg mixes from IRE (Table 1), significant differences for β-hydroxybutyric acid, lactic acid, uracil and furosine were found. It is evident that mixes are worse in terms of quality compared to shell eggs, as evidenced by the significantly higher values of all the three indices. Even if the egg products industry fraudulently utilizes IRE for the production of pasteurized egg products, our results indicate that the use of incubator-reject broken eggs is unlikely. On the other hand, sophistication through the use of IRE, which need inferior dilutions to comply with the legal limits, is a real risk. For example, considering the β-hydroxybutyric acid values, the shell eggs should be diluted nine times, while the mixes should be diluted 31 times. To respect lactic acid limits, the shell eggs should be diluted three times and the mixes five times. However, it must be stressed that some microbiological parameters, not evaluated in this research, should also be considered.

3.3 Content of β-hydroxybutyric acid, lactic acid, uracil, furosine, pyroglutamic acid, formic acid, uridine and uric acid in non-incubated eggs and IRE

The contents of the different analytical indices in the non-incubated fertilized eggs, in the fresh eggs of category A, and in the twelve IRE batches are presented in Table 2.

Table 2 Content (mean ± standard deviation) of β-OHbutyric acid, lactic acid, uracil (mg/kg ssdm), furosine (mg/100 g protein), pyroglutamic acid, formic acid, uridine and uric acid (mg/kg dm) in whole egg of two lots of non-incubated fertilized eggs, one lot of grade A shell eggs from the market (Comm) and twelve lots of incubator-reject eggs laid by hens of different ages (34, 44 or 76 weeks), from breeds Novagen White (NW) or Novagen Brown (NB), after 10 or 18 days of incubation (Time).

alt-text: Table 2

Lot	Time	Breed	Age	β-OHbutyric	Lactic	Uracil	Furosine	Pyroglutamic	Formic	Uridine	Uric
	0	NW	44	4.0 ± 0.1	nd	nd	8.7 ± 0.3	89.1 ± 9.4	135.8 ± 16.4	47.0 ± 0.2	30.5 ± 0.3
	0	NB	44	4.2 ± 0.1	162.6 ± 21.4	nd	9.9 ± 0.1	121.4 ± 23.1	279.0 ± 7.3	52.3 ± 3.5	34.1 ± 2.8
	0	Comm		4.2 ± 0.3	353 ± 12.6	nd	8.6 ± 0.7	99 ± 1.2	165.4 ± 7.8	68.3 ± 0.5	36.7 ± 0.6
1	10	NW	34	109.0 ± 6.3	3767.7 ± 29.0	nd	42.0 ± 1.8	310.5 ± 4.8	2632.1 ± 38.1	78.2 ± 1.3	43.1 ± 0.0
2	10	NB	34	53.7 ± 8.4	4919.5 ± 113.2	nd	27.0 ± 5.3	261.0 ± 4.1	3396.6 ± 124.1	67.4 ± 0.4	48.7 ± 0.9
3	10	NW	44	337.9 ± 14.6	5131.0 ± 13.6	4.9 ± 0.4	48.2 ± 0.9	535.5 ± 1.7	3032.4 ± 35.3	76.9 ± 0.9	98.2 ± 2.0
4	10	NB	44	340.1 ± 14.7	4736.2 ± 116.8	nd	49.8 ± 2.4	392.3 ± 6.3	2847.4 ± 30.8	72.1 ± 0.1	66.3 ± 1.0
5	10	NW	76	241.2 ± 0.7	3856.4 ± 43.0	1.7 ± 0.3	56.0 ± 1.7	435.4 ± 15.1	2409.4 ± 15.0	81.6 ± 0.4	73.9 ± 0.1
6	10	NB	76	20.6 ± 1.4	1387.0 ± 6.6	nd	40.1 ± 1.1	202.5 ± 1.4	963.1 ± 21.5	51.6 ± 0.0	46.9 ± 0.0
7	18	NW	34	382.8 ± 40.8	5626.9 ± 129.9	13.5 ± 0.6	45.6 ± 0.3	529.3 ± 13.6	3595.6 ± 102.0	51.6 ± 1.7	83.3 ± 3.1

8	18	NB	34	368.2 ± 40.1	3824.0 ± 2.9	21.4 ± 0.0	73.5 ± 7.3	437.0 ± 6.7	2297.6 ± 82.1	47.5 ± 0.4	78.6 ± 0.2
9	18	NW	44	442.5 ± 12.6	5822.8 ± 186.1	37.7 ± 0.7	46.5 ± 0.9	622.8 ± 16.8	3576.5 ± 129.3	6.9 ± 0.4	100.5 ± 3.2
10	18	NB	44	619.7 ± 11.1	6957.7 ± 341.7	45.1 ± 1.1	42.9 ± 2.5	639.3 ± 10.6	4547.7 ± 315.9	16.2 ± 0.5	226.0 ± 7.2
11	18	NW	76	268.7 ± 1.3	4097.2 ± 17.0	31.5 ± 1.7	63.6 ± 2.0	534.4 ± 14.4	2352.9 ± 93.1	20.6 ± 3.1	72.0 ± 0.1
12	18	NB	76	56.7 ± 5.2	1611.2 ± 131.0	nd	81.5 ± 4.2	294.6 ± 18.5	906.2 ± 59.5	70.7 ± 1.0	52.6 ± 0.5

nd: lower than the detection limit.

The legislative indices (*β*-hydroxybutyric acid and lactic acid) in non-incubated fertilized eggs and in fresh eggs were far below the established limits (10 and 1000 mg/kg dm, respectively). The low furosine concentrations are comparable to those reported for the whole egg of non-incubated fresh eggs (Hidalgo et al., 1995). The uracil is below the detection limit too, thus confirming the good hygienic quality of the eggs. The concentrations of all the compounds are similar between non-incubated fertilized eggs and fresh eggs; this result stresses the paramount role of incubation environmental conditions on anabolism/catabolism of the different metabolites in IRE. The contents of pyroglutamic acid, uridine and uric acid varied from 89.1 to 121.4 mg/kg dm, from 47.0 to 68.3 mg/kg dm and from 30.5 to 36.7 mg/kg dm, respectively, and were higher than those reported by Rossi, Pompei, and Hidalgo (1995) in fresh eggs laid by hens of the Warren and Hy-line breeds of seven different ages (23–70 weeks).

The β-hydroxybutyric acid values detected in IRE (Table 2) are well above the legal limit (10 mg/kg dm), with values ranging from 20.6 to 619.7 mg/kg dm. Furthermore, only four lots (N° 1, 2, 6, 12) had results comparable to those in the preliminary tests (Table 1) for shell eggs (21.5–136.5 mg/kg dm), while all the other lots showed values comparable to those of the mixes (163.6–542.7 mg/kg dm). The factors that most influenced the β-hydroxybutyric acid content were incubation time and hen age (Table 3). In fact, as reported by Salwin et al. (1972), during the incubation of eggs with embryo-growth stopped on day 3 the β-hydroxybutyric acid progressively increases from day 5, while in eggs with a live embryo the β-hydroxybutyric acid increases from day 7 to day 14, before decreasing until day 18, a phenomenon observed also by Uijttenboogaart et al. (1986). Additionally, the LSD test recorded a trend linked to hen age, peaking at 44 weeks (Table 2).

Table 3 Analysis of variance (mean of squares and significance) and LSD test for the contents of β-OHbutyric acid, lactic acid, uracil (mg/kg ~~ss~~dm), furosine (mg/100 g protein), pyroglutamic acid, and formic acid (mg/kg dm) in incubator-reject eggs considering as factors time of incubation, hen breed and hen age. Different letters within each factor indicate significant differences (LSD test; $p \leq 0.05$).

alt-text: Table 3

Factor	d.f.	β-OHbutyric	Lactic	Uracil	Furosine	Pyroglutamic	Formic
Time (T)	1	178919.0***	2859500***	3397.9***	1369.4 ***	141051.0***	663670***
Breed (B)	1	17398.4***	3947140***	86.6***	28.5	91550.6***	1162040***
Age (A)	2	176602.0***	17397800***	480.1***	477.4 ***	79433.9***	7222890***
TB	1	8395.2***	346368***	15.9***	851.7 ***	2018.5**	137078**
TA	2	34973.9***	891969***	349.4***	565.0 ***	5463.7***	935303***
BA	2	47366.0***	4408170***	249.5***	29.7	19123.3***	1736950***
TBA	2	3897.7***	2594440***	268.5***	325.9 ***	5845.7***	1329340***
Error	12	343.8	17925	0.4	10.4	123.7	13797
LSD test							
Incubation time							
10		183.7[b]	3966.3[b]	1.09[b]	43.8[b]	356.2[b]	2546.8[b]
18		356.4[a]	4656.6[a]	24.88[a]	58.9[a]	509.6[a]	2879.4[a]
Hen breed							

B		243.2[b]	3905.9[b]	11.09[b]	50.3[ns]	371.1[b]	2493.1[b]
W		297.0[a]	4717.0[a]	14.89[a]	52.5[ns]	494.7[a]	2933.2[a]
Hen age							
34		228.5[b]	4534.5[b]	8.73[b]	47.0[b]	384.4[b]	2980.5[b]
44		435.1[a]	5661.9[a]	21.93[a]	46.8[b]	547.5[a]	3501.0[a]
76		146.8[c]	2737.9[c]	8.30[b]	60.3[a]	366.7[c]	1657.9[c]

Significance levels: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

The lactic acid values were always higher than the legislative limit (1000 mg/kg dm), ranging from 1387.0 to 6957.7 mg/kg dm. The analysis of variance (Table 3) emphasized the influence of hen age on lactic acid content, followed by breed, their interaction and time of incubation. The LSD test (Table 2) showed a trend similar to that of β-hydroxybutyric acid, with the highest values in eggs laid by 44 weeks hens. The eggs from hens of the Novagen White breed had a higher content than those of the other breed. Finally, lactic acid increased during incubation.

Uracil (Table 2) was only detectable in two of the six batches of eggs discarded after ten days of incubation, probably for the greater hygienic attention reserved to eggs used for vaccine production. The eggs candled after eighteen days almost always had detectable levels (from 13.5 to 45.1 mg/kg dm), similar to those found in the preliminary tests (Table 1). The uracil content was mainly influenced by the incubation time, increasing progressively during storage (Table 3). The disinfection of naturally-clean eggs does not completely avoid trans-shell bacterial contamination during incubation, because of the occasional presence of dust, soil or feces (Londero et al., 2015). In fact, De Reu et al. (2006) report a significant influence of surface bacterial presence on trans-shell contamination.

IRE lots had furosine values higher than those of non-incubated eggs (Table 2) with levels from 40.1 to 81.5 mg/100 g proteins, with the exception of lot 2 which had a very low anomalous value (27.0 mg/100 g proteins). The results were similar to those of the preliminary trial (49.6–75.3 mg/100 g proteins) for shell eggs and shelled eggs. The ANOVA (Table 3) confirmed that this index is mainly influenced by incubation time and that increases on average from 43.8 (10 days) to 58.9 mg/100 g proteins (18 days) during incubation. However, the interactions between incubation time and hen breed or hen age were also important.

Pyroglutamic acid and formic acid contents ranged from 202.5 to 639.3 mg/kg dm and from 906.2 to 4547.7 mg/kg dm, respectively, much higher than in non-incubated fertilized or fresh eggs (Table 2); on the other hand, uridine levels (6.9–81.6 mg/kg dm) and uric acid (43.1–226.0 mg/kg dm) were similar to those of non-incubated eggs (on average 55.9 mg/kg dm and 33.8 mg/kg dm, respectively). These comparisons indicate the inadequacy of the two compounds as indices of IRE and advocate further investigation on pyroglutamic acid and formic acid. Pyroglutamic acid levels were significantly higher than those reported by Rossi et al. (1995; 10.6–14.6 mg/kg dm) in fresh eggs laid by hens of the Warren and Hy-line breeds of seven different ages (23–70 weeks). The pyroglutamic acid trend with hen age (Table 2) was similar to that reported by Rossi et al. (1995) in non-incubated non-fertilized eggs. The analysis of variance indicated that this analytical index was mainly influenced by incubation time and hen breed. Formic acid is instead, mainly influenced by hen age (Table 3). Pyroglutamic acid content increases in the albumen (Hidalgo, Lucisano, Comelli, & Pompei, 1996) and in the yolk (Lucisano, Hidalgo, Comelli, & Rossi, 1996) during storage of unfertile shell eggs but the levels reached after 32 days at 30 °C considering the proportions of both fractions, are really much lower (about 84 mg/kg dm) than those found in whole eggs from IRE in Table 2. Thus, the high pyroglutamic acid concentrations reached by IRE eggs may be mainly a consequence of embryonic development.

Applying the analytical protocol described by Cavanna et al. (2018) for screening purposes, *β*-hydroxybutyric acid, lactic acid, and uracil were also identified as discriminative markers on this sample set. Fig. 1 presents for each marker the comparison between the area values obtained with the HRMS screening and the amount (mg/kg dm) obtained with the quantitative target methods. Despite the use of two completely different approaches, the trend of these molecules in the samples is similar, bolstering the analytical robustness of the results.

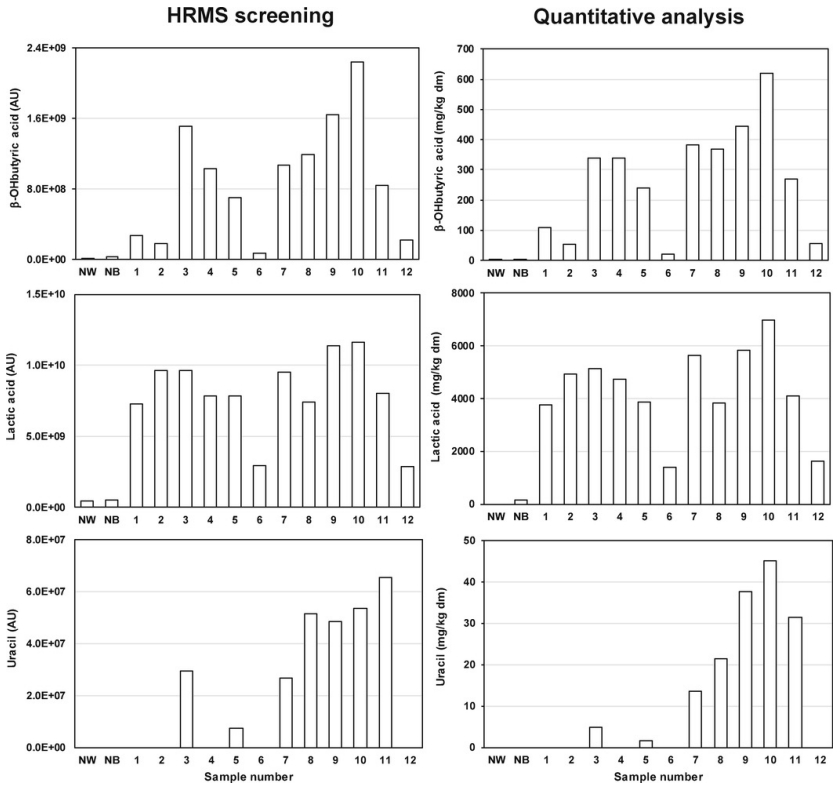

Fig. 1 Comparison between the area values (obtained with the HRMS screening) and the concentration (obtained with quantitative methods) for β-hydroxybutyric acid, lactic acid, and uracil through the samples presented in Table 2. Samples NW and NB are fertile eggs before incubation, samples 1–6 were analyzed after 10 days of incubation, samples 7–12 were analyzed after 18 days of incubation.

alt-text: Fig. 1

3.4 Effect of candling on the content of β-hydroxybutyric acid, lactic acid, uracil, furosine, pyroglutamic acid and formic acid in IRE

The IRE consists of both unfertilized eggs and eggs with early mortality of the embryo and/or eggs with weak amniotic sac, therefore a second candling selection of IRE by the egg-products industry can be hypothesized as an illegal practice to use selected IRE in egg products.

Fig. 2 shows the contents of the different analytical indices in IRE as well as in IRE divided, by candling, into two groups: eggs without apparent embryonic development (candled) and eggs with apparent embryonic development (waste). For these tests some of the lots presented in Table 2 were used.

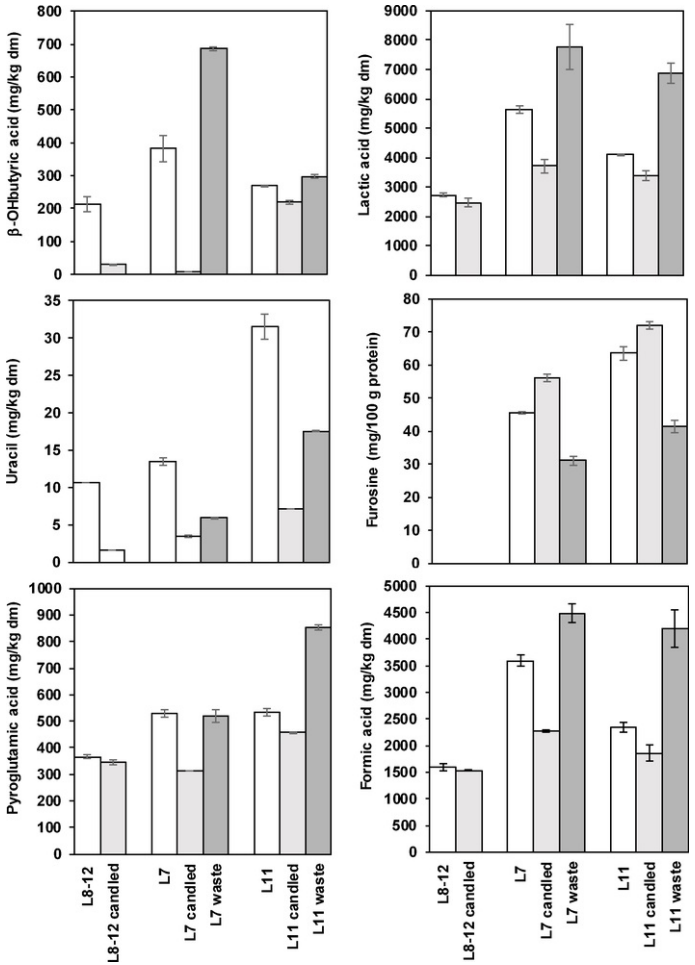

Fig. 2 Content in β-hydroxybutyric acid, lactic acid, uracil, pyroglutamic acid, formic acid (mg/kg dm) and furosine (mg/100 g proteins) in IRE non-selected, candled and discarded by candling (waste). Error bars represent the standard deviations.

alt-text: Fig. 2

The β-hydroxybutyric acid in the candled eggs was lower than in the non-selected samples for the three batches analyzed (29.3 vs. 212.5 mg/kg dm, respectively for the whole egg of lots 8–12; 8.2 vs. 382.8 mg/kg dm for lot 7; 220.2 vs. 268.7 mg/kg dm for lot 11). The operation was less effective in lot 11. As expected, higher values were found in discarded eggs. In candled eggs, the reduction of lactic acid, uracil, pyroglutamic acid and formic acid was less effective than that of β-hydroxybutyric acid.

The behavior of furosine was surprising, since candling led to higher levels in the selected eggs (from 45.6 to 56.1 mg/100 g protein for lot 7, and from 63.6 to 71.9 mg/100 g protein for lot 11) and lower values in rejects (31.1 and 41.6 mg/100 g protein, respectively). This behavior suggests a varying development of the Maillard reaction in the different substrates as a consequence of the diverse concentration of the reagents, in particular of reducing sugars. Reducing sugars are less abundant in eggs with embryo development because during the first week of incubation the carbohydrates are the primary source of energy for embryonic development, thus free glucose decreases outside and increases inside the embryo (Miraglia, 1989). Therefore, IRE candling gives lower values for all the analytical indices, with the exception of furosine.

The data of the different indices in eggs selected by candling (Table 4) show that an incubated fertilized egg may pass the control one out of five times. In fact, the egg 5 far exceeds the legal limits for β-OHbutyric acid and lactic acid and shows high values for the other parameters. The egg 1, although presenting values very similar to those of non-incubated unfertilized eggs for all parameters, slightly exceeds the legal limit for lactic acid and show

detectable uracil, suggesting a certain sensitivity of these indices to microbial contamination occurred during incubation. The values observed in the discarded eggs confirm the effective separation of fertilized eggs. Our results indicate that candling to select incubated eggs for fraudulent use by the food industry is plausible. However, the analysis of chemical indices on batches of selected eggs may allow their detection; in this context, microbial metabolites (lactic acid, uracil) seem more effective than β-OHbutyric acid. If candling is followed by an illegal dilution with sound eggs, the dilution ratio needed to respect the lactic acid legal limit is lower than that of uracil suggested limit (below the limit of detection of 0.9 mg/kg dm).

Table 4 Content of β-OHbutyric acid, lactic acid, uracil, pyroglutamic acid, and formic acid (mg/kg dm) in five selected shell eggs (Candled) and five discarded shell eggs (Waste) classified by candling in Lot 8 of incubator–reject eggs (Table 2).

alt-text: Table 4

	β-OHbutyric	Lactic acid	Uracil	Pyroglutamic	Formic
Lot 8	368.2 ± 40.1	3824 ± 2.9	21.4 ± 0	437 ± 6.7	2297.6 ± 82.1
Candled					
Egg 1	1.6	1129.7	0.90	294.8	287.7
Egg 2	2.3	746.6	nr	340.7	227.0
Egg 3	2.0	612.4	nr	325.6	250.9
Egg 4	3.6	452.3	nr	324.2	400.4
Egg 5	18.2	11848.2	33.5	643.1	7397.5
Mean ± sd	5.5 ± 7.1	2957.8 ± 4976.1	6.9 ± 15	385.7 ± 144.8	1712.7 ± 3178.6
Waste					
Egg 6	410.8	9940.7	19.5	793.6	5440.1
Egg 7	1409.4	5187.9	12.3	840.1	2777.0
Egg 8	103.5	9004.9	8.1	950.5	4598.6
Egg 9	1478.7	7847.9	8.2	741.8	4430.5
Egg 10	356.3	9660.3	23.3	868.6	4619.1
Mean ± sd	751.7 ± 643	8328.3 ± 1931.7	14.3 ± 6.9	838.9 ± 78.8	4373.1 ± 974.8

3.5 Effect of IRE dilution with fresh eggs

In addition to candling selection, it is possible to mask IRE utilization by polluting fresh eggs mixture with different IRE percentages. The results of the dilution of two different IRE lots are shown in Fig. 3. A linear trend was observed, because the content generally reflected the mass balance of the formulation. The 100% mix A and mix B showed respectively 29.3 and 219.2 mg/kg dm of β-hydroxybutyric acid, 2466.0 and 3427 mg/kg dm of lactic acid, and 1.6 and 9.2 mg/kg of uracil.

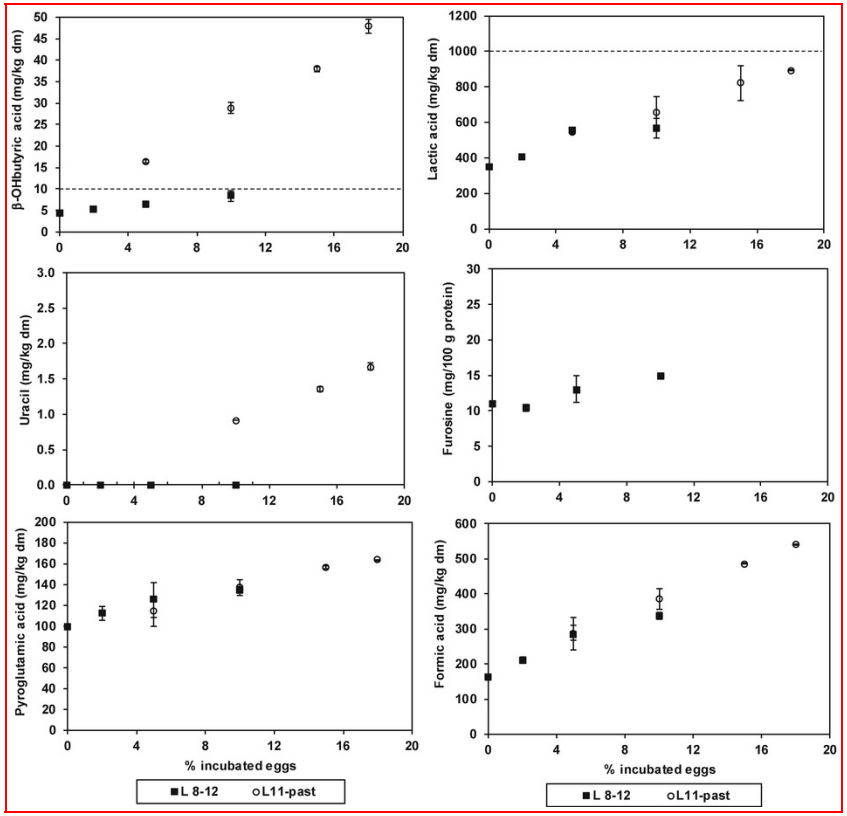

Fig. 3 Content in β-hydroxybutyric acid, lactic acid, uracil, pyroglutamic acid, formic acid (mg/kg dm) and furosine (mg/100 g proteins) in whole egg from fresh eggs diluted with 0, 2, 5, 10% IRE mix A and with 0, 5, 10, 15, 18% IRE mix B. Error bars represent the standard deviations; dotted lines indicated the legal limits.

alt-text: Fig. 3

Our results demonstrate that the danger of industries diluting egg mixtures obtained from fresh eggs with IRE without being discovered is very real; in fact, lactic acid data recorded for dilutions up to 10% and 18% were always well below the established legal limit (1000 mg/kg dm) while uracil was detectable at ≥ 10% dilution of lot B. On the other hand, β-hydroxybutyric acid content was higher than the legislative limit (10 mg/kg dm) at all the dilutions of lot B, while for lot A was lower at dilutions up to 10% (8.4 mg/kg dm). Dilution of candled IRE also led to low furosine values, while formic acid in diluted samples was still higher than in non-incubated fertile eggs.

4 Conclusions

In conclusion, the analysis of IRE laid by hens of different ages, breed and incubation time showed β-hydroxybutyric acid concentrations (20.6–619 mg/kg dm) higher than the established legal limit (10 mg/kg dm), confirming it as an excellent marker of embryonic development. Lactic acid, always found in high concentrations (1387.0–6957.7 mg/kg dm), was a less sensitive analytical index considering the limit set by the legislation (1000 mg/kg dm). Uracil was generally detectable in batches candled after 18 days of incubation, while was absent in almost all batches candled after ten days and in fresh and non-incubated fertilized eggs. Furosine (42.0–81.0 mg/100 g proteins) higher than in non-incubated eggs (8.6–9.9 mg/100 g proteins), confirmed its reliability as eggs freshness index. Pyroglutamic acid and formic acid contents were markedly different between non-incubated fertilized or fresh eggs and IRE, but before considering their possible use as a marker for IRE identification the natural variability in fresh eggs should be assessed. On the contrary, uridine and uric acid are not suitable indices for IRE detection.

The results show that IRE candling and/or dilution with non-incubated eggs are possible fraudulent practices and can lead to egg products that meet legal limits. Thus, to guarantee greater consumer protection it would be advisable to lower the legislation thresholds of β-hydroxybutyric acid (from 10 to 6 mg/kg dm) and lactic acid (from 1000 to 600 mg/kg dm). Furthermore, uracil could be suggested as a future additional legal parameter, considering as a warning signal concentrations ≥0.9 mg/kg dm, the detection limit of our method.

Declarations of interest

None.

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Highlights

- Fraudulent use of incubator-reject eggs (IRE) in egg products was investigated.
- Candling and/or dilution with sound eggs masked the use of IRE in egg products.
- Lower limits of European legislative indices (β-hydroxybutyric acid and lactic acid) are needed.
- Uracil is suggested as a future additional legal parameter.
- A non-target screening HRMS confirmed these three markers as the most discriminant.

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