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Comparison of two extraction methods for ergosterol determination in vegetal feeds

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SUMMARY

Ergosterol is the principal sterol of fungi in which it plays an essential role in cell membrane and other cellular constituents. This sterol is considered as a good marker of fungal contamination and of mycotoxin production. After validation of ergosterol quantification by HPLC-UV system (linearity range: 0.2 to 20.0 mg/ml, repeatability: 3.27%, between day precision: 4.75%), 2 extraction methods of ergosterol from 3 vegetal matrixes (maize, barley and wheat) were compared: the first one, normalized by the AFNOR, is based on solid phase extraction (SPE), while the other is based on liquid/liquid extraction (LLE). The LLE procedure allowed ergosterol extraction gains of around 20% for high initial sterol contents (3 to 5 mg/kg) in naturally contaminated matrixes or in spiked samples, and of 86% for low initial sterol contents (1-2 mg/kg) in maize. Moreover, the precision of ergosterol determination was comparable for the 2 methods even if it was slightly lower using LLE and was more affected by the initial ergosterol contents in vegetal matrix than by its nature. These results suggest that ergosterol contents in vegetal feeds would be underestimated with the official method (SPE) and emphasize the importance of the extraction step.

Keywords : Ergosterol, Extracting method, HPLC, Solid phase, Liquid/liquid phase.

RÉSUMÉ

Comparaison de deux méthodes d'extraction pour le dosage de l'ergostérol dans les matières premières végétales

L'ergostérol est le stérol principal des moisissures et joue un rôle essentiel au niveau de la membrane et d'autres constituants cellulaires. On considère celui ci comme un bon marqueur de contamination fongique et pourrait être un indicateur du risque mycotoxique. Après validation de la quantification de l'ergostérol par un système HPLC-UV (gamme de linéarité : 0,2 à 20.0 mg/ml, répétabilité : 3.27 %, reproductibilité : 4.75 %), 2 méthodes d'extraction de l'ergostérol à partir de 3 matrices végétales (maïs, orge et blé) ont été comparées. La première méthode normalisée par l'AFNOR [2], repose sur l'extraction en phase solide (SPE), la seconde met en oeuvre l'extraction liquide/liquide (LLE). La procédure LLE a permis des gains d'extraction d'ergostérol d'environ 20 % pour des niveaux de contamination en stérol de 3 à 5 mg/kg dans les matrices naturellement contaminées ou dans des échantillons supplémentés, et de 86 % pour des concentrations de 1 à 2 mg/kg dans le maïs. La précision de la quantification de l'ergostérol est comparable avec les 2 méthodes, même si elle est légèrement plus faible avec la LLE et a été plus affectée par les concentrations d'ergostérol initiales dans la matrice végétale que par la nature de celle-ci. Ces résultats suggèrent que le contenu en ergostérol dans les matrices végétales est sous-estimé avec la méthode SPE et soulignent l'importance de l'étape d'extraction.

Mots-clés : Ergostérol, Mycotoxines, méthode d'extraction, HPLC.

Introduction

Molds are common contaminants of agricultural commodities, foods and feeds. Fungal development on alimentary substrates can lead to different detrimental effects: alteration of technological properties, decrease of nutritive value, and synthesis of mycotoxins… [15]. Evaluation of molds development is of interest to estimate global quality of raw materials and may be useful to take decision on their possible use.

Ergosterol is considered as the principal sterol of fungi and it plays an important role as cell membrane component [23]. Therefore, it has been proposed as a global indicator of mycological quality of foods and feeds [4, 5, 18, 20, 21]. One interest of this compound is that it is not affected by harsh physical treatment, allowing the detection of previous molds contamination [12]. Consequently ergosterol levels are commonly used as quality parameters in ecological [6], industrial [7], and agronomics environments [8, 16]. Moreover, significant correlations were found between ergosterol and the major mycotoxins (fumonisin B1, Zearalenone, Deoxynivalenol, Ochratoxin A, patulin) in maize [14], rice [17], tomato [9] and wheat [1]. Therefore, ergosterol determination can be considered as a good index of fungal development on cereals and could be an early indicator of potential mycotoxin production. Its determination can be used in industry to screen productions, prior to mycotoxin analysis. On cereals, 3 μg of ergosterol per gram is considered as the maximum acceptable level for maize while for wheat, 8 μg of ergosterol per gram is the retained value for certifying correct quality of the grains [5]. On the other hand, when the amounts of ergosterol are upper than 8 μg/g on maize and 12 μg/g on wheat a doubtful quality of grains is suspected [5]. Moreover, associations between high ergosterol contents in feed and lowering performance on guinea fowl and ducks have been reported [3].

For these reasons, several chromatographic methods have been proposed to assess ergosterol in crops including recently both gas chromatography–mass spectrometry [13] and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry [6]. Most of them are based on UV absorption of ergosterol and SEITZ *et al.* [20] used reverse phase High Pressure Liquid Chromatography (HPLC), which becomes the reference method in France [2]. Prior to the quantification, a saponification is performed to release

esterified ergosterol from cytosolic lipid particles. This hydrolysis allows total ergosterol quantification (free ergosterol from fungi walls and cellular/cytoplasmic ergosterol stemming from ergosteryl esters). Total ergosterol amount is usually considered as the most sensitive marker of fungal biomass [20]. After this saponification step, an extraction/purification procedure by solid phase extraction is required by some methods [2] whereas other authors described the use of a liquid/liquid purification/extraction [4]. Some discrepancies on results obtained by different techniques show that it is really important to elaborate or modify actual analytical techniques for quantifying ergosterol rapidly with sufficient accuracy.

The aim of this study is to compare the yield of these two extraction/purification procedures on maize, wheat and barley to determine what should be the best screening method for crops.

Materials and Methods

CHEMICALS AND REAGENTS

Ergosterol standard (5,7,22-ergostatrien-3β-ol; EC n° 200-352-7, purity min. 90%) was purchased from Sigma. A stock solution at 1000 mg/L in dichloromethane/isopropanol (99.5/0.5) was diluted with the same solvent to obtain standard solutions ranging from 0.5 to 20.0 mg/L. Stock solution was stored at -20°C whereas standard dilutions where kept at 4°C for one week. All solvents used for extraction, clean up and chromatography were of HPLC grade and purchased from ICS (Toulouse, France). SPE Extrelut 20 cartridges were purchased from Merck (Darmstadt, Germany).

SAMPLE EXTRACTION PROCEDURES

Samples (5 kg) of maize, wheat and barley from France, with no trace of mold development macroscopically detectable were first grinded in meal. Then, meal sub samples of 15 g were used for analysis.

Extraction of ergosterol was performed using the method of SCHWADORF and MULLER [19] with modifications according to the AFNOR norm NFV 18-112 [2]. Briefly, 15 g of meal, 45 mL of methanol, 15 mL of ethanol, 6 g of KOH, and 60 μL of pyrogallol (10% in methanol) were refluxed for 30 minutes at 80°C. The mixture was cooled to 20°C and filtered through fluted paper.

Solid Phase Extraction (SPE)

Three mL of the saponified mixture were applied on the Extrelut® column. After 20 minutes, ergosterol was eluted by 15 ml hexane. The eluate was then evaporated in the dark under a gentle stream of nitrogen. The dry residue was dissolved in 1 mL of dichloromethane before HPLC quantification.

Liquid/Liquid Extraction (LLE)

Fifteen mL of the saponified mixture were extracted twice with 30 mL of petroleum ether. This extract was then washed twice with acidifed water (2 mL H_2SO_4 95% in 1 L of distilled water, pH 1). The extracted mixture (3 mL) was then evaporated in the dark under a gentle stream of nitrogen. The dry residue was dissolved in 1 mL of dichloromethane before HPLC quantification.

HPLC PROCEDURES

Chromatographic system is composed by a M 2200 pump (Bischoff, Leonberg, Germany) connected to a Lichrospher Si 100, 5 μm , 250 x 4.6 mm (Bischoff, Leonberg, Germany). The UV detection was monitored by a Spectra-focus (Spectra physics, USA). The chromatograms obtained were monitored by PIC 3 software (ICS, Toulouse, France). Both standard and samples (20 μL) were eluted by dichloromethane/isopropanol (99.5/0.5). Flow rate was 1.1 mL/min. Ergosterol peak was detected at λ = 282 nm. Ergosterol was quantified in samples by peak area measurement and comparison with peak area obtained for standard solutions.

Standard solutions were used to perform a regression study between the observed area and the injected quantity of ergosterol. Five concentrations were injected in triplicate to check the linear range and estimate the detection limit on standards. Repeatability was assessed with a same standard solution (5 mg/L) which has been injected ten folds successively. The same standard solution (5 mg/L) was used over a long period of time to assess the "between run" precision. Recovery was assessed on maize meal (n = 6) spiked with 5 mg/kg of pure ergosterol regarding the same maize without spiking (n = 8).

STATISTICAL ANALYSIS

Results obtained with the two extracting methods were compared by comparison of means (Student t test). Differences were considered as significant when the p values were less than 0.05.

Results and discussion

VALIDATION OF HPLC PROCEDURES

In our experimental conditions, retention time of ergosterol was about 7 minutes (fig 1A).

Linearity

The detected response (as measured by peak area in μv x s) was strongly correlated with ergosterol concentration for all over the range of the tested concentration (from 0.2 to 20.0 mg/L, r = 0.995 with a slope of 250000 and passing by the origin). The limit of detection was estimated at 2 ng ergosterol injected on the column (Table 1). Another statistical complementary approach was performed to confirm linearity. The variation coefficient of the response factor (response/ concentration) was calculated for each concentration tested (Table 1). Since the variation coefficient of the response factor is less than 10%, we can consider that the response of the detector is linear for ergosterol concentrations ranging from 0.2 to 20.0 mg/L [11].

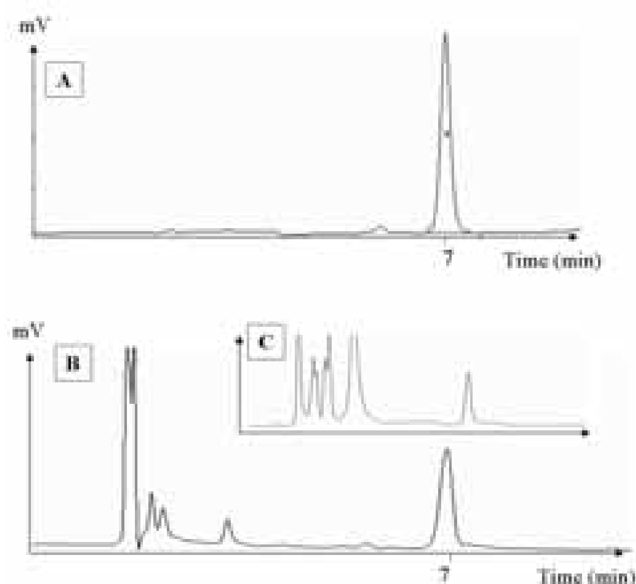

FIGURE 1: **A**: Chromatogram of a 20 mg/L standard solution of ergosterol.
B: Chromatogram of a maize sample extracted with Solid Phase Extraction (SPE).
C: Chromatogram of a maize sample extracted with Liquid/Liquid Extraction (LLE).

Repeatability and "between day" precision

One standard solution (0.5 mg/L) was injected ten times successively in the chromatographic system. The percentage of variation obtained on the peak area recorded was 3.27% (Table 1). The same standard was injected each day during one week period (n = 6). The percentage of variation obtained on the peak surface was 4.75 % (Table 1). During this study, we noted that the diluted standard solution was not stable with time when stored at 4°C in the dark. Indeed, a decrease of 20% was recorded on peak area after two weeks and this diminution reached 50% after three weeks (data not shown).

COMPARISON OF THE TWO EXTRACTION PROCEDURES

Recovery of ergosterol

No interfering peak was seen on chromatograms obtained after SPE or LLE of the same saponification extract (fig. 1B, 1C). Higher peaks were obtained with LLE in front of the chromatograms signalling a lower specificity concerning apolar substances compared to SPE. They did not interfere with interpretation of chromatograms because they are in earlier retention time zone (before 7 minutes).

Since finding meal or grain without ergosterol is impossible, ergosterol content was firstly measured in one batch of maize considered as mildly contaminated with molds (n = 8). This batch was further used for recovery determination assays by spiking it with pure ergosterol at 5 mg/kg (n = 6). The mean percentage of recovery obtained by iteration with the sigma plot software in maize supplemented with 5 mg of ergosterol /kg was 83 ± 28% with the SPE method and 102 ± 9% with the LLE. The value obtained with the SPE was in agreement with those generally reported (around 90%) [2, 4, 10, 19].

The two extraction methods were performed using the same saponification mixture prepared from the same spiked sample. For each determination, ergosterol content was always higher with the LLE procedure than with the SPE one. Albeit the standard deviations greatly varied, they remained comparable with those already described for repeatability, variation coefficients (VC) ranging from 5 to 21% in the SPE method [2]. Moreover, since data were obtained during several days, they also were informative on between days reproducibility. The only available results concerning reproductibility of the SPE method were those of an inter-laboratory study. They revealed important differences with deviations in results ranging from 10 to 55% [2].

Determination of ergosterol in samples

Together maize, wheat and barley were tested for ergosterol content. For each raw material, six samples of the same batch were analysed. After the saponification of the meal, ergosterol of each sample was extracted using either SPE as described in the official method or for the LLE procedure. The retention time of ergosterol was around 7 minutes whatever the extraction method and chromatograms in this zone were quite similar (fig 1B. 1C).

Table 2 presents the comparison of the two extracting methods on the different matrixes. Whatever the studied matrixes (maize, barley or wheat), the average ergosterol contents as well as almost all the individual sample values obtained after LLE (n = 6) were always significantly higher than those obtained after SPE in official method ($p<0.01$ for maize and barley, $p<0.05$ for wheat). The ergosterol LLE gain reported to SPE was 86.1 ± 30.3%, 21.9 ± 9.0% and 21.0 ± 0.2% for maize, barley and wheat respectively. The coefficients of variations ranged from 5% to 20% with SPE and from 9 to 31% with LLE depending on the matrix and ergosterol contents. The higher values of the VC (respectively 20 and 31% for SPE and LLE) were obtained for maize in

Repeatability	Between run precision	Detection limit	Linearity	Range of linearity
5.0 mg/L	5.0 mg/L	mg/L	r	mg/L
n = 10	n = 6 (one week)			n = 3
VC* = 3.27 %	VC* = 4.75 %	0.1	0.995	0.2 to 20.0

* VC: Variation coefficient

TABLE 1: Validation parameters of the method of ergosterol determination by HPLC-UV.

which the ergosterol content was the lowest (1.23 and 2.32 mg/kg for SPE and LLE respectively). As the repeatability and the reproductibility of the HPLC quantification step were respectively 3.27% and 4.75%, the great variations in ergosterol recovery were probably related to the saponification and/or the extraction steps. Because high variation coefficients were observed with the two extraction procedures, the decrease of precision for ergosterol determination would probably due to the low ergosterol content in this matrix rather than the matrix nature, i.e. maize. Indeed, the same VC (20%) for the same contamination level (around 1.5 mg/kg) was obtained with the official method (SPE) for different matrixes [2]. Intermediate coefficients of variations (8% with SPE and 10% with LLE) were recorded from wheat in which the highest ergosterol content was found (4.26 and 5.11 mg/kg for SPE and LLE respectively). These results were in agreement with those already recorded from wheat with the standardized method (6.2% repetability and 9.7% reproductibility) for an ergosterol contamination level of 9 mg/kg and were comparable to those obtained from barley for the same contamination level [2]. Consequently, the moderate decline of precision was linked to the high ergosterol content and probably not to a matrix effect. As far as the barley matrix was concerned, low coefficients of variation (5% and 9% for SPE and LLE respectively) were observed for moderate ergosterol amount (3.38 and 4.12 mg/kg for SPE and LLE respectively). Again, this relative high precision degree was associated with the sterol concentration instead of the matrix nature. Indeed, the official method exhibits the same coefficient of variation for the same contamination level (around 3-3.5 mg/kg) for different matrixes (maize: 5%, wheat 6.2%) but no data were available for barley [2].

Taken together, the precision degree of ergosterol determination in the vegetal feeds is essentially dependant on the initial ergosterol amounts and matrixes effects seem to be minor.

Conclusion

The Liquid-Liquid extracting/purification method (LLE) seems particularly adapted for studying ergosterol in various matrixes. Indeed, the ergosterol quantification by HPLC analysis exhibits gains of extractions reaching 20 % on wheat

Matrix	Ergosterol content (mg/kg) according to the 2 extraction methods SPE	LLE
Maize *(n = 6)*	1.23	2.32
Mean	1.15	2.33
Median	0.25	0.72
SD*	20	31
VC* (%)	31	86.1 ± 30.3
Gain LLE/SPE (%)		
P	< 0.01	
Barley *(n = 6)*		
Mean	3.38	4.12
Median	3.36	4.00
SD*	0.18	0.38
VC* (%)	5	9
Gain LLE/SPE (%)		21.9 ± 9.0
P	< 0.01	
Wheat *(n = 6)*		
Mean	4.26	5.11
Median	4.27	5.05
SD*	0.36	0.53
VC* (%)	8	10
Gain LLE/SPE (%)		21.0 ± 0.2
P	< 0.05	

*VC: Variation coefficient; SD: Standard deviation

TABLE 2: Determination of total ergosterol by HPLC-UV on a batch (n = 6) of maize, barley and wheat. After saponification step, a liquid/liquid extraction (LLE) was compared to a solid phase extraction (SPE) on the same extract. Results were expressed in mg/kg of meal.

and barley compared to the SPE procedure. A gain of 86 % was even found on maize, but on this matrix, the low level of ergosterol in tested samples (1 mg/kg) may contribute to these great differences between the 2 extraction methods. It seems that for such low concentrations the whole saponification/ extraction/determination may not be very reproducible whatever the method considered (variation coefficients ranging from 20 to 30%). The deficit of ergosterol extracted by the SPE procedure could be explained by irreversible adsorption of ergosterol on the solid phase as already described for other compounds as organophosphorus on SPE columns [22].

Since the precision (variability) of each method is quite similar and seems to be more dependent on ergosterol levels (inversely proportional) than on matrixes, this study demonstrates that Liquid-liquid extraction of ergosterol is a simple and cheap method, particularly adapted for ergosterol screening from many vegetal matrixes.

Finally this study tends to demonstrate that ergosterol levels are probably underestimated with the official method that could partially explain some discrepancies that are sometimes reported between ergosterol quantification and molds numeration and/or mycotoxin analysis of raw materials [5, 12].

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